

How to relax with a hamburger

A proposed new agency is a welcome first step towards a recovery of public trust in food. But an unsatisfactory history and unresolved questions necessitate continuing vigilance.

Earlier this week, a federal courthouse in Texas became the setting for a bizarre libel case. In the dock is the prominent and outspoken US talk-show host Oprah Winfrey, who told her audience during a live broadcast two years ago that concern about the possible presence of bovine spongiform encephalopathy (BSE) in US cattle had "stopped me cold from eating another hamburger". Challenging her, under relatively recent legislation passed in Texas and several other states, is a group of cattle ranchers who claim that Winfrey should be made to pay for the lost beef sales that followed her remark.

The outcome of the case will have both legal and political implications. At its heart is the same issue as that which the UK government has sought to address with its publication this week of proposals for a new Food Standards Agency (see page 313): how to encourage open public debate on food safety — including the most recent scientific findings on the topic, however tentative — without unfairly jeopardizing the interests of the producers.

The United Kingdom's recent BSE crisis, to which the creation of the new agency is partly a response, showed up in a dramatic fashion the extent to which the balance had got out of hand. Assurances of the safety of British beef in the late 1980s had more to do with preserving the economic interests of a lucrative industry than with solid science. Most pernicious, perhaps — as the recently announced public inquiry into the crisis is likely to reveal — is the way that these interests became directly reflected in the tight control exercised by the Ministry of Agriculture, Fisheries and Food (MAFF) over the analysis and interpretation of all veterinary data related to the epidemic of BSE as it swept through UK herds.

From this perspective, the arrangements for the new agency can be warmly welcomed as representing several important steps in the right direction. For example, the decision that it should be formally

independent of government, and report to the Secretary of State for Health, embodies a principle that advice on food safety will now be prepared at arm's length from the interests of food producers. Equally welcome is the government's commitment to transparency: the commission's advice will be published, allowing the government's response to such advice to be closely scrutinized.

Still unresolved, however, is the question of how the agency's research agenda is to be set. Science will play a large role in its activities; the agency will have a research budget of about £25 million (US\$40 million) a year, and the government has promised that its assessments of food standards and safety will be "based on the best available scientific advice". This is certainly a necessary condition for rebuilding public confidence in the safety of British food products. There are already belated but encouraging signs of increasing input into the planning of food research from outside government, which will include representatives of the consumer. But these conditions will lead to success only if the agency has the right leadership. The US Food and Drug Administration provides a model in this respect at least, having been led in recent years by a political appointee who communicated well and who earned the trust of many political and public interests.

In setting up the Food Safety Agency, the UK government has set the stage for a bold experiment to tackle safety in a manner free (in principle) from the shibboleths of the past. Coinciding with other key decisions it faces, such as the future of the Institute of Food Research, as well as of MAFF's Central Science Laboratory, this experiment presents the government with a unique opportunity to forge a research base responsive to the long-term needs of food producers and consumers alike. But the new agency is still some years away. In the meantime, MAFF will need to be kept under close scrutiny. □

Halt the xeno-bandwagon

Xenotransplantation's risks make a moratorium essential.

The momentum towards clinical trials of xenotransplantation is seemingly unstoppable, powered as it is by new prowess in genetic engineering that promises to pulverize the barrier of cross-species rejection, blended with an otherwise insatiable need for transplantable organs and multimillion dollar investment by biotechnology companies (see page 320).

But overcoming the huge obstacles to cross-species rejection is much further off than some biotechnology companies would like their investors and the public to believe. And premature trials carry the risk of creating new human diseases and pandemics (see page 327). Regrettably, the debate about xenotransplantation has amounted to a cacophony of messages from the various stakeholders about the potential risks and benefits. How can the public be expected to evaluate the possibilities when the experts themselves cannot agree?

Regulation of human cloning is politically easy, given its restricted benefits and the practical difficulties. Much greater polit-

ical courage and enlightenment will be required to clarify the complex and pressing question of how best to regulate xenotransplantation, and this time in the face of predictable opposition from industrial and patient constituencies. Problems must not be papered over through compromises between the various conflicting interests, brokered by bureaucrats and expert committees within regulatory agencies, as has been amply shown by the bovine spongiform encephalopathy crisis, and the contamination of haemophiliacs with HIV during the 1980s.

A well-organized and informed public debate should precede any action by regulatory agencies. One model for the latter, offered on page 326, deserves studious attention. But until action is taken in this direction, politicians would do well to err on the side of caution, and agree on an international moratorium on clinical trials. What is at stake is not only risks to public health, but the real promise of xenotransplantation, which can only be compromised by undue haste. □



Universities warned on danger of new links to weapons labs

[WASHINGTON] US universities should reconsider their willingness to work closely with the nuclear weapons laboratories under the so-called 'strategic alliances' programme, according to the Natural Resources Defense Council (NRDC). The influential Washington-based lobby group says that such efforts could lead indirectly to the development of new nuclear weapons.

The programme, which started last summer, seeks to engage the universities' best brains on issues of interest to the weapons laboratories, without allowing the laboratories' secrets to leak out to the open environment of the universities.

But a report by the pressure group, which has been prominent in recent years in analysing and criticizing the US nuclear weapons programme, argues that the five major centres established under the programme are engaged in work that will help the United States to develop new and more powerful nuclear weapons in the future.

In particular, a draft of the report, obtained by *Nature*, says that work on shock physics at one of the centres — the California Institute of Technology (Caltech) — will pose a risk of proliferation, making it easier for aspiring nuclear powers to develop advanced nuclear weapons without having to test them.

In a move that reflects concern inside the weapons laboratories about the programme, foreign students and staff at the university centres have already been barred from having



Superbrain: universities will gain access to the world's most powerful supercomputers, such as this one recently installed at Sandia.

direct access to the laboratories' supercomputers (see below).

Critics say the unequal treatment of foreign students and staff reflects the ambiguous position of the whole strategic alliances pro-

gramme, and that the programme implicates the universities in the development of weapons of mass destruction.

But the programme's defenders argue that research at the centres is not nuclear weapons research as such, and that helping the weapons laboratories with their new Science-based Stockpile Stewardship programme for maintaining the weapons without testing will enable the United States to comply with the Comprehensive Test Ban Treaty (see *Nature* 387, 541; 1997).

The five centres were set up last summer after a fierce competition between teams at forty research universities. The winners — Caltech, Stanford University and the universities of Chicago, Utah and Illinois — will each receive \$25 million in research support over five years, with the strong prospect of a five-year extension.

They should also get access to the world's most powerful massively parallel supercomputers, now being developed and installed at the three weapons laboratories — Lawrence Livermore in California and Sandia and Los Alamos in New Mexico — under the Department of Energy's (DOE's) Advanced Strategic Computing Initiative (ASCI).

Although some universities originally drafted proposals which they considered of direct relevance to the weapons programme, the DOE has dissuaded them from this approach, asking them to simulate complex dynamic systems that are distinct from nuclear weapons. The energy department

Ban on access for foreign scientists poses a problem for partnership

[WASHINGTON] Whatever the ethical merits of the 'strategic alliances' programme (see above), a ban on the participation of foreigners — who make up as many as half of the hundreds of research students and staff working at the five university centres involved in the programme — indicates the practical challenges the DOE faces in operating it.

The partnership programme was conceived mainly to restore the supercomputing expertise of the weapons laboratories. Senior laboratory officials admit that their pre-eminence in this field in the 1970s and 1980s has waned with the development of the new, massively parallel computers, which are best understood by

young computer scientists outside the laboratories.

Staff and students at the university centres fall into three categories — US citizens, permanent residents and other foreigners. As in most US university computer science and engineering departments, about half the staff and students are non-citizens. Many of the faculty are permanent residents — holders of 'green cards' — and are protected by US law against being treated differently from US citizens. Most of the students are not permanent residents.

The DOE has introduced a short-term policy, to remain in force until April, under which no foreign national is allowed access

to the laboratory supercomputers or the codes that run on them. According to Michael Heath, head of the Illinois centre, the temporary policy "can't hold in the long run, or the DOE will be in violation of the law".

Heath says the current situation reflects a difference in perspective between technical staff in the laboratories, who want to open up access, and security officials, who are resisting change in their established procedures.

Matthew McKinzie, the main author of a report drawn up by the National Resources Defense Council, says that the ban on access by foreigners reflects resistance in the intelligence divisions at the

laboratories — especially Lawrence Livermore — to the partnership programme, which he says was "imposed on the laboratories" by the DOE.

The energy department is now reviewing the ban. "They understand that it is an issue for all of the centres, and are working hard to solve the problem," says Daniel Meiron, head of the Caltech centre.

Gilbert Weigand, the DOE's deputy assistant secretary for strategic computing, says the students may be accommodated by giving them access to an unclassified supercomputer facility, which he declined to specify, outside the weapons laboratories.

C.M.

hopes the universities' involvement will help scientists at the weapons laboratories to solve the central technical problem of stockpile stewardship — the full computer simulation of a thermonuclear weapon's operation.

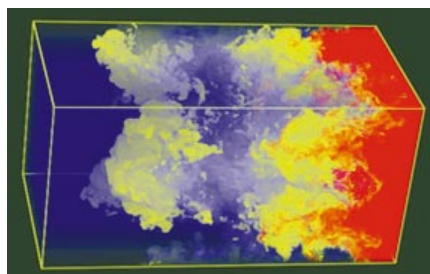
The University of Illinois centre, for example, will study solid-fuel rockets, while Chicago simulates astrophysical thermonuclear flashes. The Utah team will investigate accidental fires and explosions as these impinge on nuclear weapons safety, and Stanford will look at turbulence in gas-turbine engines.

But the Caltech centre will investigate the dynamic response of various materials to the detonation of high explosive — a problem of clear relevance to nuclear weapons design. According to the NRDC draft report, Caltech's proposal included the study of the behaviour of beryllium, uranium and actinide metals under shock from high explosives.

"If the Caltech program is permitted to continue for its five to ten year course, much of the work behind generating a bomb code will have been accomplished," says the NRDC.

Caltech officials now say that these materials will not be included in their study. "We will not be working on nuclear weapons materials, or on anything that is a heavy metal," says Daniel Meiron, head of the Caltech centre. Steve Koonin, provost and vice-president of research at Caltech, adds that he would "not allow research to be done at Caltech" for the nuclear weapons programme.

The NRDC argues, however, that the shock physics to be carried out at Caltech is exactly what proliferators would require if they wanted to advance from a primitive gun-



Dual use: simulations of explosions are relevant to both 'shock physics' and weapons design.

type nuclear device to an implosion-based device, or even a hydrogen bomb boosted by a thermonuclear secondary, without testing.

In contrast to the vehement criticism of research related to President Ronald Reagan's Star Wars initiative fifteen years ago, campus reaction to the arrival of money from the nuclear weapons programme has so far been subdued. David Pershing, the head of the Utah centre, says that despite strong anti-nuclear feeling in the state (which is downwind of the Nevada site where US weapons tests took place), critics have accepted that his team is working on safety, not on building nuclear weapons.

At Caltech, however, the 100-strong Southern California Federation of Scientists has attacked the partnership in local newspapers, and called on David Baltimore, the new president of Caltech, to take a public position on the issue. Baltimore declined to be interviewed for this story, referring questions to Koonin.

The NRDC wants a government review of the proliferation implications of the partner-

ships with the universities, and a debate within the universities on whether they should be involved. Its draft report says that the universities' acceptance of the programme "represents an obvious — but financially fortuitous — failure to comprehend the full scope of the current nuclear weapons programme".

The report argues that the \$4.5-billion-a-year stockpile stewardship programme is less concerned with complying with the test ban treaty than with circumventing it, by developing computer models so powerful that the United States will be able to improve its nuclear weapons without testing.

The DOE has consistently denied such charges. But it does accept that the stockpile stewardship programme will help the United States to maintain its nuclear weapons design capability indefinitely, in case new weapons are required in the future, and that part of the function of the partnerships is to help the laboratories recruit scientists for that purpose.

A spokesman for Livermore referred questions about the partnerships to the DOE. But Tom Adams, an ASCI project leader at Los Alamos, says that staff there are enthusiastic about partnerships. "There's a lot of excitement," he says. "People are most anxious to interact with the universities."

Gilbert Weigand, deputy assistant secretary for strategic computing at the DOE, declines to comment on the NRDC's criticisms until its final report is published. But he stresses that the partnerships "are completely unclassified projects", and adds: "They are things the universities wanted to do, in their own best interests."

Colin Macilwain

Switzerland seeks to head off ban on use of transgenic animals

[MUNICH] The Swiss government is trying to pre-empt the outcome of a national referendum calling for major restrictions on genetic engineering — including a ban on the use of transgenic animals — by presenting its own proposals for strengthening the rules governing the use of such techniques.

Known as the 'Gen-Lex' motion, the initiative aims to coordinate existing legislation covering a range of activities relating to genetic engineering, and to close any apparent gaps. Scientists, environmentalists and animal rights groups have been asked for their comments.

A national ethics committee is also to be set up in the next few months to address issues relating to the use of animals in genetics research. One of its tasks will be to assess whether proposed experiments using transgenic animals are ethically justified.

The referendum, scheduled for June, was initiated in 1992 by pressure groups opposed to biotechnology. Although widely supported by environmentalists and animal rights activists, it has been strongly opposed

by many scientists, who warn that its approval would seriously harm biomedical research in Switzerland, and could persuade pharmaceutical companies to relocate elsewhere (see *Nature* 388, 315; 1997).

"A general ban on the use of transgenic animals would be disastrous for many Swiss researchers," says Franco Cavallo, director of the Institute for Oncology in Bellinzona and a socialist member of the Swiss parliament.

Switzerland has no specific regulations on genetic engineering, as this is considered to be covered by a variety of laws in areas such as environmental and animal protection.

A recent survey has shown that three-quarters of the population are opposed to constitutional bans, such as that proposed on the use of transgenic animals. The Gen-Lex motion seeks to build on this aversion to bans by tightening laws designed to ensure that abuses cannot occur.

Although the referendum has firm support from many people, the outcome of the vote is still in doubt. To succeed, it requires the majority of the country's 26

cantons to vote in favour. This is unlikely, according to Cavallo, because there is relatively little opposition to genetic engineering in the French- and Italian-speaking parts of Switzerland.

Cavallo also argues that the Gen-Lex motion is too abstract and complex to persuade supporters of the referendum to change their minds. But Peter Mani, a microbiologist who heads the department of gene technology and society at the University of Zürich, is optimistic that the initiative will help to prevent approval of the measures being put to the referendum by reassuring the public that genetic engineering will be strictly controlled. "The opponents have gone too far in their demands," he says. "Complete bans make the public uneasy."

He believes that, barring a major accident or other event, time and national conservatism are on the scientists' side. "The Swiss are not likely to choose a radical solution unless some unpredicted scientific disaster happens shortly before the referendum," he says.

Quirin Schiermeier

Japan gears up to debate brake on human cloning

[TOKYO] Japan's Council for Science and Technology, the principal science policy-making body which is chaired by the prime minister, is setting up a committee to discuss a possible legal ban on human cloning.

The move was announced last week and coincided with the signing by 19 European countries of a convention in which they agreed to introduce legislation to ban human cloning for reproductive purposes (see *Nature* 391, 219; 1998).

The Japanese committee will be made up of experts in ethics, medicine and the law, and plans to hold its first meeting in February. It aims to reach an agreement on its recommendations by May, in time for the G7 meeting of the leaders of the advanced industrialized countries to be held in Birmingham, in the United Kingdom, at which issues raised by human cloning are scheduled to be discussed.

Keiichi Tanigaki, director-general of Japan's Science and Technology Agency (STA), said at a meeting of the council last week that the cloning of humans is "a fundamental issue in bioethics", and that an "open and nationwide debate" is needed in Japan before a decision is made on how it should be controlled.

But critics point out that the committee's meetings will be closed to the public, in contrast to similar meetings at ministries such as the Ministry of Health and Welfare.

The issue of human cloning "requires careful and determined consideration" says Norio Fujiki, an emeritus professor at Fukui Medical School and one of Japan's leading bioethicists. "The first step requires the involvement of people from different fields, so that extensive discussions can take place."

Since last year's birth in Scotland of Dolly, the cloned lamb, the council has repeatedly asked Japanese researchers in the public and private sectors to hold back from carrying out research into human cloning. The STA and the Ministry of Education, Science, Sports and Culture (Monbusho) have responded by announcing plans to limit their funding and research in this area until proper guidelines have been laid down.

A protocol protecting human embryos already exists in Japan; donating eggs is illegal under ethical rules laid down by the Japanese Society of Obstetrics and Gynaecology. But there are loopholes. It was recently revealed that a Japanese couple had agreed to donate fertilized eggs to an American couple, and that there have been more than 100 cases of such 'adoption' from Japan. Although this practice is prohibited in Japan, it is legal in the United States.

Asako Saegusa

UK's food standards agency will report to health minister

[LONDON] The UK government revealed its plans for an independent food standards agency last week, responding to pressures that have built up steadily since the recent crisis over bovine spongiform encephalopathy (BSE) and other food safety scares.

The agency will be created by a merger of the food safety, research and nutrition divisions of the Ministry of Agriculture, Fisheries and Food (MAFF) and the Department of Health, and will have the power to enforce food safety "from plough to plate".

Although about 80 per cent of its staff of several hundred will come from MAFF, it has been decided — after what is said to have been an intense internal battle in Whitehall — that the agency will report to the health minister. It is expected to be set up by the middle of next year, after further public consultations and parliamentary debate.

In a white paper (policy document) setting out its proposals, the government says the agency will be headed by a chief executive and 12 commissioners, most of whom will be drawn from public and consumer interests. Its £100-million (US\$150 million) annual budget will be met from a levy on the food industry, and the agency will also take charge of the agriculture ministry's annual £25-million food safety research budget.

The announcement has been welcomed by consumer groups, such as the Consumers Association, and by many food scientists. The proposals are based almost entirely on a report written by Philip James, director of the Rowett Research Institute in Aberdeen, at the invitation of the Prime Minister, Tony Blair, in March last year, when he was still in opposition. Another of James's proposals — that there should be increased public representation on government expert advisory committees — is already being implemented.

At the core of the white paper is the decision to take responsibility for food safety away from MAFF. The potential for a conflict of interest in a single ministry representing the interests of both producers and consumers of beef had been flagged by observers for some years.

At the white paper's launch in London last week, Jack Cunningham, the agriculture minister, acknowledged that "people had long argued that this [arrangement] was flawed and not in the public interest".

The issue came to a head during the late 1980s and early 1990s with MAFF's apparent reluctance to take stronger action to protect consumers from eating meat infected with BSE, because of pressure from farmers who feared that further protective measures would lead to industry losses.

Measures were belatedly taken when scientists announced the possibility that eating BSE-infected meat may have caused the neurodegenerative disorder Creutzfeldt-Jakob disease (see *Nature* 380, 273; 1996).

The decision to place food safety research in the hands of the agency reflects a view that the influence of the food industry lobby may have obstructed open and transparent publication of the agriculture ministry's research. Indeed, some critics, such as Erik Millstone, a food policy researcher at the Science Policy Research Unit at the University of Sussex, fear that MAFF may still find it difficult to relinquish control over both food safety and research to the food standards agency.



James: proposals are based on his report.

Between now and the launch of the agency, all new research proposals will be looked at by the independent expert committee that advises the government on food science. But its main food and agriculture research laboratory — the Central Science Laboratory — is to remain under MAFF's control, suggesting that the ministry is not about to let go.

Millstone says that he is also worried about a passage in the white paper that says the agency "will be required to consult agriculture ministers" when considering new measures to be added to the Food Safety Act.

While the agency retains powers to override agriculture ministers, Millstone believes that this will be difficult in practice, as the agency's commissioners may well be "too intimidated" to ignore ministerial advice. He also questions the constitutional basis for "unelected officials overriding elected members of the government".

But the view that the continued involvement of MAFF represents bad news for research is not shared by all researchers. Bronek Wedzicha, head of the department of food science at the University of Leeds, for example, says he has never been prevented from publishing research funded by the agriculture ministry.

Wedzicha says that scientists need to understand that the scientific civil service operates in a different way from mainstream science. "If you have a finding, don't act recklessly, insisting on the right to publish anywhere you like," he says. "Reason with them, and they'll come round to your point of view."

Ehsan Masood

'Group debate' urged for gene studies

[WASHINGTON] A US presidential commission is likely to recommend soon that researchers should lose the liberal access to anonymous tissue samples that they now enjoy for genetic studies whose outcomes could affect whole groups of people.

The National Bioethics Advisory Commission (NBAC) is drawing up recommendations on informed consent and the ethical use of tissue samples that are due to be made public within a few months. It is likely to recommend that researchers be required to carry out 'community consultation' before initiating a trial that could put groups at risk.

The move follows the identification last year of a genetic mutation that predisposes to colon cancer and is carried in 6 per cent of Ashkenazi Jews — Jews of European descent who make up nearly all American Jewry. During the study, 766 archived, anonymous blood samples were used that were originally collected during screening for Tay Sachs disease.

The samples are among at least 283 million archived tissue samples that US scientists rely on for their work in genetics, epidemiology, pathology and other fields.

But critics are claiming that the findings, by a team headed by Bert Vogelstein of Johns Hopkins University in Baltimore, Maryland (see *Nature Genetics* 17, 79–83; 1997), have created job and insurance risks for Ashkenazim. The findings follow the earlier identification of the most common mutation that predisposes to breast and ovarian cancer. That mutation in the BRCA2 gene, also identified using Tay Sachs samples (see *Nature Genetics* 14, 188; 1996), is carried in just over 1 per cent of Ashkenazi Jews.

"The community is being clearly pinpointed," says Ezekiel Emanuel, a former member of NBAC, who heads the depart-

ment of clinical bioethics at the National Institutes of Health.

Local ethics boards, known as institutional review boards, which approve research protocols before scientists are allowed to proceed, would probably be the enforcers of 'community consultation' introduced at the urging of the NBAC. Alternatively, tissue banks might set up panels to identify protocols requiring such consultation, an approach proposed by the National Action Plan on Breast Cancer.

The community consultation recommendation emerged from the genetics subcommittee of NBAC, and is to be debated — along with other proposals about informed consent for the use of tissue samples — by the full commission at a meeting in Los Angeles on 5 and 6 February. The commission tentatively plans to post its tissue sample recommendations on the Internet in interim form next month (www.nih.gov/nbac/nbac.htm).

The commission's recommendations would not be binding. But its advice has in the past carried weight. Last spring, for example, its suggestions on cloning were immediately written into a bill sent to the Congress by President Bill Clinton.

Researchers have reacted warily to the latest proposal. "This is a Pandora's box unless they very carefully define what a community is, because it could easily be misinterpreted by regulators," says Mark Sobel, chief of the molecular pathology section at the National Cancer Institute and president-elect of the Association for Molecular Pathology. "And then it would be a major burden on researchers."

Sobel argues that there would be difficulties in defining a 'community' — ought bald men, for example, be consulted before the launch of a study on the role of androgen

receptors in baldness? Questions would also arise as to who legitimately represents such a community. For example, do Orthodox, Conservative or Reform rabbis speak for Jews? "We don't need an extra official layer" of protection, says Sobel, who argues that review boards are already sensitive to issues such as community risk. "These things do not get done without time, people and money."

Kenneth Offit, chief of clinical genetics at Memorial Sloan-Kettering Cancer Center in New York, and a member of the team that identified the most common mutation that predisposes to breast and ovarian cancer, says that community consultation before experiments is unrealistic for researchers in population genetics. In the course of their careers, researchers may look at dozens of mutations, not all of which yield significant results.

It would be "hard to imagine" how in practice to hold a community conference before each experiment for a mutation "which is of unclear significance", says Offit, who was also a member of Vogelstein's team. In the worst case, he adds, onerous new requirements "could shut down a very important aspect of genetic epidemiological research: population genetics".

But others argue that the recent explosion of genetics research has changed the moral landscape, making individual consents for the use of tissues in research inadequate in cases in which groups such as the Ashkenazim are so keenly affected by discoveries.

"Every time we change our ethical standards, somebody says 'This is going to be more red tape,'" says Patricia Barr, a lawyer who headed a subcommittee that drafted tissue sample recommendations for the National Action Plan on Breast Cancer. But "the way we now think about these issues, the individual's consent is not always sufficient".

Community consultation "upholds other important social values, so I don't see the same risk" as the researchers, says Lori Andrews, a law professor at Chicago-Kent College of Law. "There are other very important values — not least of which is the trust in the research enterprise — at stake here."

The NBAC would not be alone in invoking 'community consultation', which is now used, for instance, by the Food and Drug Administration for trials in emergency situations in which individuals are unable to consent. And the National Heart, Lung and Blood Institute relies heavily on community consultation for genetic studies in African American and Native American communities, says Teri Manolio, who manages community-based studies of genetic risk for the institute.

"You can't go back to the community after you have the results and say 'Oh, by the way, we'd like to consult with you'. It has to start from the very beginning." **Meredith Wadman**

SUSI digs deep for distant galaxies

[LONDON] Keen to keep pace with the Hubble Space Telescope, astronomers at the European Southern Observatory (ESO) have produced their own ground-based colour image of far-distant objects (right) which ESO describes as being "unique in covering four bands with an image quality better than one arc-second".

The image, produced by the Superb Seeing Imager (SUSI) Deep Field project of the 3.5-metre New Technology Telescope (NTT) at La Silla, Chile, involves 122 frames in four colours — blue, green-yellow, red and near-infrared — of an area of 'empty sky' just south of the celestial equator.

ESO admits that a shorter exposure time and brighter sky background, caused by light emission in the upper atmosphere, mean that the image is not as deep as that



produced by Hubble. But its high quality is said to justify the construction of the Very Large Telescope (VLT) in Chile, which is due to start operation within a few months, and one of whose goals will be to perform spectroscopic analysis of distant galaxies.

Poll reveals backing for xenotransplants

[PARIS] More than three-quarters of the US public would consider a xenotransplant — the transplant of an animal organ into a human — for a loved one “if the organ or tissue was not available from a human”, according to a poll carried out on behalf of the US National Kidney Foundation (NKF). Only 2 to 5 per cent would completely rule out a xenotransplant in a life-or-death situation (see Briefing, page 320).

But widespread ambivalence and ignorance about the subject was revealed elsewhere in the poll, which was carried out by the market research company Southeastern Institute of Research, based in Richmond, Virginia. Although two-thirds of the 1,200 people polled think that xenotransplantation research should continue, more than three-quarters had only “heard a little” about xenotransplantation, and only 15 per cent had “heard a lot”.

Opposition to xenotransplantation was strongest among the best informed. But one-quarter of those strongly opposed to xenotransplantation research, and half of those who had never heard of it, would still consider a xenotransplant if no alternative were available.

The poll revealed a marked difference in the issues of most concern to the public and to scientists. Twenty-seven per cent were concerned about organ compatibility, but only 13 per cent about risks from infectious diseases, the major concern among scientists (although more than three-quarters said that learning more about disease risks could change their feelings about xenotransplants).

Respondents preferred baboons or chimpanzees as donors rather than pigs, whereas



non-human primates are widely considered unsuitable and more risky by xenotransplant scientists (see page 324).

Polls of public attitudes on xenotransplantation need to be interpreted with considerable caution, says Abdullah Daar, a transplant surgeon at the Sultan Quaboos University in the Sultanate of Oman, and chairman of the xenotransplantation advisory committee set up last year by the World Health Organization. Daar points out that, although many polls have shown wide public support for xenotransplantation research, they fail to distinguish between research and clinical trials.

“How xenotransplantation is ‘sold’ to the public is likely to influence the response,” says Daar. Responses will differ depending on whether xenotransplantation is billed “as a ‘last resort’ or as the ‘best option’”. Public attitudes could also change quickly, says Daar, predicting that a combination of “a

perceived inadequate scientific base and negative publicity” would be likely to lead to a moratorium.

Strikingly different results from those of the NKF poll were obtained in the recent Eurobarometer poll of 15,000 people in the European Union, where support for xenotransplantation was assessed in the context of the acceptability of genetic engineering. More than three-quarters of respondents supported genetic engineering for the production of drugs and vaccines, but only 36 per cent thought xenotransplantation was morally acceptable and should be “encouraged”, and the technology was considered the most risky of modern biotechnology applications (see *Nature* 387, 845; 1997).

The National Kidney Foundation has no position on xenotransplantation, according to Ellie Schlam, one of its officials. Schlam says the survey was carried out “so NKF could act as a catalyst to initiate a discussion among the American public”. The report on the survey recommends targeting information campaigns at the clergy, who emerged from the poll as the profession with the most influence on the issue.

Most of those polled — including agnostics — said that endorsement of xenotransplantation research by religious leaders could make them agree to it. “In order to have them [clergy] making a positive, rather than negative, impact, they need to be fully informed... preferably before any public communication effort on xenotransplantation,” recommends the report.

Louisa Chapman, an official at the US Centers for Disease Control in Atlanta, who has been involved in organizing consultation about imminent US guidelines on xenotransplantation, admits that the public lacks knowledge about the issue. “The debate has largely been in scientific, government and medical circles, but outside the communities working on those there is not much public awareness of the issues, which are mainly discussed in fairly obscure scientific literature, and commercial and research circles.”

Indeed, organizations such as Friends of the Earth and the European Consumers’ Association, which have been prominent in debates about the safety of genetically modified organisms, have not yet taken a position on xenotransplantation. Strong views on the subject in Britain, for example, tend to be confined to animal rights groups such as Compassion in World Farming.

“I’m very surprised by the nature of the public debate,” says David Onions, a xenotransplantation researcher at the University of Glasgow. Onions describes the infectious disease risk as “a very serious matter”, and adds: “I’m surprised it has not been more of an issue.”

Declan Butler

FDA ‘fails to keep track of transplant patients’

[WASHINGTON] The US Food and Drug Administration (FDA) lacks an adequate means of locating tissue transplant recipients if donor tissue is discovered to be infected, according to the General Accounting Office (GAO), the investigative arm of Congress. And tighter regulations that the agency is proposing fail to address the problem adequately.

A report on human tissue banks issued by the GAO in December concludes that the FDA is seriously hindered in protecting public health because it has no register of the 400-or-so US tissue centres which process

several hundred types of human tissues. Fewer than half of these centres are accredited by industry associations.

The FDA “can neither notify all tissue facilities as potential public health threats arise nor plan inspections from a complete registration of tissue facilities”, says the report. In one typical case, researchers were unable to track the recipient of a tissue graft infected with hepatitis C.

The FDA has proposed a broad strengthening of its oversight of tissue transplantation, to be implemented slowly over coming years. It plans to

require all tissue centres to register with the agency, and to report “adverse events”. But the GAO report says that, in failing also to require centres to report “errors and accidents,” the agency “is missing an opportunity to target facilities that may need additional oversight”.

For instance, the FDA recently discovered while inspecting one company that it had distributed corneas before receiving test results showing that one cornea had tested positive for HIV. Under the agency’s proposed plan, such an event would not have to be reported. **Meredith Wadman**

Brussels' subsidy to shift away from infrastructure

[MUNICH] European Commission proposals aimed at encouraging poor countries and regions to spend more of their European Union (EU) subsidies on research, but placing less emphasis on building new facilities, will be considered in Brussels this week.

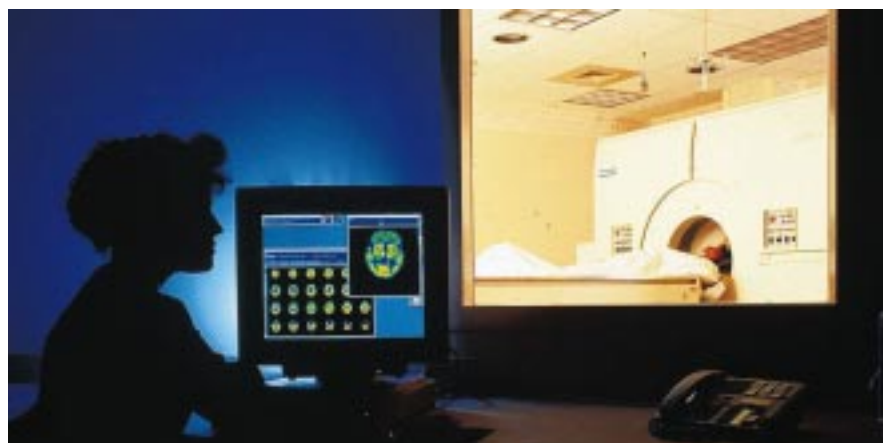
If accepted, the recommendations — which are in line with broader EU policy reforms on 'economic and social cohesion' due to be introduced after 2000 — will be presented to the European Parliament and Council of Ministers for discussion.

The subsidies, known as 'structural funds', will continue after 2000 with the aim of promoting competitive development in poor regions (see *Nature* 385, 192–193; 1997). But regions and countries eligible for the funds will be encouraged to spend a higher proportion on research than they do now. They will also be encouraged to spend more of the funds allocated for research on training, innovation, technology transfer and dissemination of results.

The building of infrastructure facilities has previously been generously funded. Structural fund spending on research has increased to 5.7 per cent of the total from 3.9 per cent in the early 1990s, but the technology gap between poor and rich countries and regions has closed only slightly.

The new round of structural funds, covering the period 2000–2006, will total ECU275 billion (US\$297 billion), including ECU45 billion earmarked for new EU members. The proportion dedicated to research is likely to be comparable to the budget of the commission's fifth Framework programme for research, which will be about ECU16 billion (see below). And the new philosophy on structural funds complements that on the latest Framework programme, which renews emphasis on the dissemination and exploitation of results.

Alison Abbott



Brain invaders: could cerebral imaging techniques such as PET scanning pose a risk to privacy?

Advances in neuroscience 'may threaten human rights'

[PARIS] Neuroscience is being increasingly recognized as posing a potential threat to human rights, just as another area of biology — research in human genomics — may lead to an excessive focus on genetic determinism and raises the spectre of genetic discrimination. This was one of the conclusions to emerge from the annual public meeting of the French national bioethics committee held last week in Paris on the theme of 'Science and Racism'.

Jean-Pierre Changeux, the chairman of the committee and a neuroscientist at the Institut Pasteur in Paris, told the meeting that understanding the working of the human brain is likely to become one of the most ambitious and rich disciplines of the future.

But neuroscience also poses potential risks, he said, arguing that advances in cerebral imaging make the scope for invasion of privacy immense. Although the equipment needed is still highly specialized, it will become commonplace and capable of being used at a distance, he predicted. That will open the way for abuses such as invasion of personal liberty, control of behaviour and

brainwashing. These are far from being science-fiction concerns, said Changeux, and constitute "a serious risk to society".

Denis Le Bihan, a researcher at the French Atomic Energy Commission, told the meeting that the use of imaging techniques has reached the stage where "we can almost read people's thoughts".

The national bioethics committee is taking such threats so seriously that it is launching a study to consider the issues and recommend possible precautions. The study will also cover more immediate issues such as the legal question of whether criminals are responsible for their actions; Changeux predicts an increase in defence arguments based on irresponsibility due to a genetic predisposition to certain types of behaviour.

In closing the meeting, Claude Allègre, the minister for national education, research and technology, hinted at the creation of a revamped parliamentary office of technology assessment, arguing that the national bioethics committee's approach in the life sciences needed to be applied to other areas of science.

Declan Butler

Framework themes grow to four, but budget stays the same

[MUNICH] The European Commission has increased the number of themes in its next five-year Framework programme for research from three to four, but is retaining the original budget figure.

The addition of a theme to the programme, which is due to start next year, is a response to proposals from the European Parliament (*Nature* 391, 3; 1998).

The parliament had also proposed a small increase in the budget of the fifth Framework programme (FP5). But the final proposal, released in Brussels last week and to be presented shortly to member states as

represented by the Council of Ministers, sticks to the original budget of ECU16.3 billion (US\$17.6 billion.)

In addition, the parliament had suggested making terminology more straightforward, but the commission rejected this and will keep its less-precise naming tradition.

Thus the names suggested for the four thematic programmes are: 'improving the quality of life and the management of living resources'; 'creating a user-friendly information society'; 'promoting competitive and sustainable growth'; and 'preserving the ecosystem'. The commission

does not want to separate energy and environment in the last-named programme, although the parliament had asked that each of these have its own budget line.

The commission also added two more targeted programmes-within-programmes — 'key actions' — to a list it had hoped to limit to 16. The additions are 'the ageing population' and 'global environmental change and climate'.

The commission's revised proposals are due to be discussed by the research ministers of the 15 member states, meeting as the Council of Europe, in mid-February.

A.A.

World Bank renews its support for Brazil

[SÃO PAULO] The World Bank has approved the third phase of Brazil's Programme of Support for Scientific and Technological Development (PADCT III), which seems to have overcome recent funding problems.

PADCT functions through matching loans from the World Bank, but financial pressures previously prevented the country's Ministry of Science and Technology from passing on funds to researchers. The scheme ran into particular problems in the early 1990s during the administration of the now-impeached president Fernando Collor de Mello. Matters reached a low point for PADCT in 1992; at one point, because of its failure to provide local funds, the programme was using only 3.8 per cent of the World Bank loans.

Since then, however, the situation has improved dramatically, and the programme has become a major source of funding for many high-level research teams. It also funds attempts to remedy some of Brazil's worst regional imbalances in research support, which is currently concentrated in the southeastern states of São Paulo, Minas Gerais and Rio de Janeiro.

The latest PADCT programme also encourages the private sector to increase its spending on research, showing the influence of Brazil's science minister, Israel Vargas. Initially, PADCT III will cost US\$360 million. As before, \$155 million will come from the Brazilian government and an equal sum from the World Bank. But this time, the private sector will supply the remaining \$50 million. PADCT III is planned to end in 2001, with a total budget by then of \$600 million.

PADCT was initiated in 1984 as a way to complement the Brazilian government's internal funding for science. Although it focuses primarily on applied research, the programme also funds basic science, and particularly the importation of new procedures and techniques.

During the first phase, which lasted from 1985 to 1989, 1,800 different projects were financed by PADCT at a cost of \$166.8 million. The spending planned for the period 1990–95, on 820 projects, was \$156.4 million. A further \$143.6 million was spent in 1994–95, \$67.3 million coming from the World Bank and the rest from the Brazilian government.

But with Brazil facing heavy inflation, steering the resources through the labyrinthine Brazilian bureaucracy became a major hurdle. In particular, problems in securing local matching funds created widespread frustration, as scientists were unable to obtain money for projects that had been approved.

Things have changed since the 1992 nadir. Although red tape is still a problem, it no longer threatens research projects as it previously did. And the start of the new programme coincides with a new spirit of confidence in many parts of the Brazilian scientific community.

For example, the country has just started to operate fully a synchrotron laboratory that took several years to build, and several teams of foreign scientists are already visiting the facility (see *Nature* 390, 328; 1997). A supercomputer devoted to weather studies has been so productive that a second is to

be bought, for installation probably later this year. And a record number of cooperation projects with foreign researchers are being carried out, ranging from tropical ocean studies to space crystallography aboard the Space Shuttle.

The only significant embarrassment has been the failure to launch the country's second indigenous scientific satellite on a domestically designed and built rocket, the VLS, last November (see *Nature* 390, 10; 1997). But the failure may have been more the result of bad luck than bad design, according to a report released recently.

One of the four motors of the rocket's first stage failed to ignite properly because of a fault in the device that should have detonated the propellant electrically. But a sophisticated attitude control mechanism worked perfectly, correcting the angle of the rocket after the motor had failed to ignite.

PADCT, with the World Bank's support, has played an important role in boosting morale in the research community. According to Ricardo Brentani, director of the São Paulo branch of the Ludwig Institute for Cancer Research, one major benefit is that the selection of projects is based strictly on merit, with explicit guidelines helping scientists to understand the criteria by which their requests will be judged.

The guidelines also help to prevent bias among the referees. And one interesting by-product, says Brentani, is that the questionable qualities of some prominent researchers have been exposed, and a new generation of qualified researchers all over the country acknowledged. **Ricardo Bonalume**

Nanjing tops China's league for international science citations

[TOKYO] Nanjing University in southern China is still the country's most productive source of scientific papers and has increased its lead over China's two top universities in Beijing, according to *Science Citation Index* figures.

The SCI's statistics, from the US-based Institute of Scientific Information, show that Nanjing, which has long emphasized basic research, published 570 SCI papers in 1996, 118 more than in the previous year.

For several years, Nanjing University has outperformed Peking and Tsinghua universities, in the northern capital of Beijing, in output of SCI papers (see *Nature* 378, 543; 1995). And this gap has widened: Peking and Tsinghua, which rank second and third respectively, each produced just under 300 papers listed in the citation index, and increased their output only by a few tens of papers during 1995 (see table).

The figures on the numbers of SCI-listed

papers produced in 1996 by universities and research institutes in China were released last month by the Chinese State Science and Technology Commission and the Chinese Institute of Scientific Information in an annual exercise that looks also at the

Papers published by Chinese universities in international journals in 1996*

University	Number of papers
1. Nanjing	570
2. Peking	285
3. Tsinghua	273
4. Chinese University for Science & Technology	270
5. Fudan	230
6.(jointly) Jilin	167
6.(jointly) Lanzhou	167
8. Nankai	162
9. Zhejiang	160
10. Shandong	122

*As listed in the *Science Citation Index*

number of engineering and domestic (Chinese-language) research papers.

Nanjing's comparatively high output — which remains far behind the thousands of SCI papers typically produced by leading universities in the West — results from a policy of encouraging basic research. In the 1980s, the university's president Qinyue Qu, an astronomer, introduced bonuses for researchers who published SCI papers funded by local industry.

Officials at Peking and Tsinghua universities are sceptical about Nanjing's performance. They argue that it is merely the anticipated outcome of a highly targeted approach aimed at producing more research papers in international journals, and that numbers of SCI papers do not necessarily indicate the general quality or standing of a university's research output. Nevertheless, they admit that their output of SCI papers is low and needs to increase. **David Swinbanks**

FDA 'has the power to police human cloning experiments'

[WASHINGTON] The US Food and Drug Administration (FDA) argued last week that it already has authority to regulate — and possibly prohibit — experimental attempts to clone humans. Spokesman Larry Bachorik says the agency “could apply to human cloning the same authority it has used for the past decade to regulate cell therapy and gene therapy”, with a view to “protecting the safety of patients and the public health”.

Bachorik says Richard Seed, the Chicago physicist who has proposed human cloning, would have to meet the same requirements as any investigator proposing a new drug for human use, because nucleic acids and manipulated cells are considered biological products, and all biological products regulated by the FDA are considered drugs (see *Nature* 391, 218; 1998). These requirements would include making an investigational new drug application.

Westinghouse pulls out of prize sponsorship

[WASHINGTON] The administrator of the Westinghouse science prize — a high-profile award given to 40 high-school science

students each year — says he is “100 per cent confident” that a new sponsor will be found for the award following the announcement last week by Westinghouse that it plans to end its 57 years of sponsorship in 1999.

Once a famous electrical engineering conglomerate based in Pittsburgh, Pennsylvania, Westinghouse bought the CBS television network two years ago, and subsequently divested itself of most of its engineering interests. It says the popular school science award — which attracted 1,600 project entries this year, including some from every US state — is no longer relevant to its business interests.

But Peter Bennett, president of Science Service, the non-profit group that administers the prize, says his organization “has had its door beaten down” by would-be sponsors prepared to spend \$1 million a year to support the award. He expects to have a new sponsor in place in the spring.

Stone Age man goes 'home' to Italy

[MUNICH] Ötzi, the only Stone-Age human to be found outside a burial plot, was last week given a police escort to his final resting place, a climate chamber simulating glacial conditions in the museum of archaeology built specially for him in Bolzano in northern Italy.

Ötzi, who is more than 5,000 years old, was found in 1991 in a glacier near the Ötzi valley in the Alps, close to the Austrian–Italian border. At first it was believed that his body lay in Austrian territory, and he was therefore taken to Innsbruck University, where he was subject to extensive research for six years. But his nationality was disputed, as it was found that he had been discovered exactly 92.6 metres south of the border on Italian territory.

The Austrians were reluctant to let him return home, and feelings have been running so high that the authorities judged police protection to be necessary on Ötzi's drive home because of fears of attack by Austrian nationalists.

Russia's young scientists set to escape the draft

[MOSCOW] At its first meeting of the new year, the Russian cabinet instructed Vladimir Fortov, the minister for science and technology, to suggest measures to improve the situation of Russian science. One plan is to grant exemption from military service to young scientists graduating from the country's leading universities.

“A very large number of scientists will not report to the barracks because of the government decree soon to be signed,” Fortov said after the cabinet meeting. The

move reflects the fact that the average age of Russian scientists is between 50 and 55, and scientific institutions are estimated to need between 10,000 and 12,000 young researchers between the ages of 22 and 25.

Medical research cuts bite hard in Canada

[MONTREAL] Canada's Medical Research Council, the major federal agency granting money for health research, revealed last week that it has been able to fund only one in five grant applications because of continuing budget cuts. Only 15 per cent of applications for new projects and 41 per cent of renewal requests were approved.

And all successful applications received less than three-quarters of the funding that the council's peer review committees recommended. Medical researchers have expressed concern that young researchers, unable to find funds, will migrate to the United States. The research council has seen its budget fall by 13 per cent in recent years.

Revamp of Italy's grants system makes its mark

[ROME] The news last week that three-quarters of all applications were turned down in Italy's newly introduced university grants system has, ironically, been widely

greeted as a positive sign that Italy is on the way to adopting European norms of peer review. Leading Italian scientists have long criticized a system of grant allocation notorious for distributing small amounts of money to virtually all applicants to avoid offending anyone.

Despite satisfaction with its increased selectivity, however, the management of the new system is still seen as problematic. It is run by only five people, all of whom have to cover fields that are too wide to manage. And money remains in a single pot, requiring subjects as far apart as medieval history and astrophysics to compete directly with each other.

But Carlo Calandra, president of the Italian Institute for Physics and Condensed Matter and one of the five 'wise men', says he is happy with the system, and that it needs only a little fine-tuning.

In the next round, applications will have to be submitted in both English and Italian so that foreign referees can be consulted, the first time this has happened in Italy.

Don't desecrate the Moon, say Indians

[BOSTON] A group of Navajo Indians is protesting about the decision by the US space agency NASA to allow the Lunar

Prospector, which is orbiting the Moon, to carry a capsule containing an ounce of the ashes of Eugene Shoemaker, the planetary scientist and lunar geologist who died last year (see *Nature* 391, 221; 1998).

The ashes were put on the space probe as a tribute to a scientist who contributed greatly to knowledge about the Earth's only natural satellite. But the Indians are claiming that the Moon is sacred ground that should not be desecrated with human remains. NASA officials have apologized, saying they did not intend to cause offence, but pointing out that they are unable to keep the craft from crashing, ultimately, on the lunar surface.

'Guinea pig' Glenn heads off to space again

[WASHINGTON] Astronaut-turned-US Senator John Glenn, who has flown only once in space for a total of five hours in 1962, will get a second chance. After more than two years of intense lobbying, the 76-year-old space hero finally persuaded the NASA administrator, Daniel Goldin, to let him join the crew of the space shuttle *Discovery* in October.

Glenn's joyride is a minor windfall for two scientific teams, who will use him as a test subject for already approved studies of how increased stress influences protein metabolism in space and how the hormone melatonin affects sleep.

Last chance to stop and think on risks of xenotransplants

US regulations are soon to be released allowing trials of animal-to-human transplants. Some feel this is premature, arguing that the risks of creating human diseases remain uncertain, and more preclinical research is needed.

Prospects that the transplant of animal organs, tissues and cells into humans will become a practical proposition are looking increasingly promising, as progress is made towards overcoming the formidable barriers of cross-species rejection. Few doubt that xenotransplantation could eventually bring important medical benefits. But there is still heated debate about the circumstances under which it should be allowed to cross the Rubicon from animal studies into the clinic — if at all.

The dilemma is that, when one tests animal-to-human transplants, one is also carrying out another, unwanted, experiment — testing the remote but real danger that animal viruses might jump to humans and cause man-made pandemics. This concern has come to the fore over the past year just as earlier concerns about animal welfare and the ethics of xenotransplantation have faded (see *Nature* 382, 197 & 380, 6; 1996).

Optimism that breeding disease-free animals might overcome viral risks has been dealt a blow recently by the discovery that pigs, the current donor of choice, harbour endogenous retroviruses (PERV) that can infect human cells *in vitro*. Multiple copies of PERV are integrated in the pig genome which suggests that breeding 'clean' pigs will be extremely difficult, if not impossible.

The discovery was made independently by virologists Robin Weiss, at the Institute of Cancer Research in London (see *Nature* 389, 681; 1997), and David Onions, at the University of Glasgow. It has already prompted the US Food and Drug Administration (FDA) to call a moratorium on porcine transplants until trials can build in adequate tests to screen for the viruses in donor organs, and to monitor for them in the recipients afterwards.

Weiss and Onions' work makes it

"painfully evident" that the understanding of the risks is still evolving, said Mary Pendergast, the then senior adviser to the commissioner of the FDA, at a meeting of the agency's advisory sub-committee on xenotransplantation last month. This made development of regulations "difficult," she added.

Regulation of xenotransplantation in the United States is at a turning point with the imminent release of guidelines that will give the go-ahead to clinical trials. The guidelines are expected to be published soon after a public meeting organized in Washington this week by US Public Health Service (PHS) agencies, including the FDA, the National Institutes of Health, and the Centers for Disease Control and Prevention (CDC).

US pre-eminence in medical research means that the guidelines will inevitably influence the development of xenotransplantation internationally (see box, page 322). Moreover, as infectious diseases do not respect national borders, US actions may have consequences for the rest of the world.

The new guidelines will be more stringent than a draft version put out for consultation in 1996. They will give the FDA oversight of all trials, instead of leaving this to local institutional review boards as originally proposed. The creation of a federal xenotransplantation advisory committee along the lines of the Recombinant DNA Advisory Committee is also being explored.

This tougher tone has been welcomed by many, including the American Society of Transplant Physicians (ASTP), which had criticized the earlier guidelines for failing to provide sufficient public health safeguards. The society said the proposal to leave oversight to institutional review boards was a recipe for disaster, arguing that the boards had a narrow view of the issues that could be detrimental to broader public concerns.

Trials might open Pandora's box

The emphasis on the risk of zoonosis — the transmission of animal diseases to humans via organ transplants or blood — is relatively recent. Louisa Chapman, an expert on xenotransplantation at CDC, recalls that

when the issue was first raised at CDC in 1993 her first thought was "why would we spend taxpayers' money on that, as it's only once in a blue moon that someone puts a baboon or liver heart into someone, they die within 72 hours and that is the end of the story?"

Past transplants of animals' organs into humans have been so rare and unsuccessful as to have never been considered a serious public health issue. But, as the prospect that the techniques may become a clinical reality moves closer, many fear that, as the number of organ recipients grows, so too will the risk to the human population. "We are at the cusp of a possible explosion of xenotransplantation efforts," says Pendergast.

Commercial interest is already strong. The Swiss company, Novartis, the main corporate player in xenotransplantation, is prepared to invest up to US\$1 billion in the technology in the near term. And the market may be worth up to \$6 billion in 2010, estimates Peter Laing, an analyst at Société Générale Straus Turnbull in London, who predicts that Novartis could account for more than half.

But there is a broad consensus that substantial preclinical research is needed before xenotransplantation is likely to succeed in the clinic, and that more time is needed to study the nature of the risks. And some scientists are worried that the proposed regulations may herald a premature and dangerous acceleration of clinical applications.

What is mainly fuelling this momentum is the shortage of human donor solid organs, such as hearts, kidneys, lungs and livers. But the numbers of patients involved at present is relatively small in public health terms (although a reliable source of organs could greatly expand use of transplants). Most scientists also believe that, whereas success in implanting animal cells may be within reach, the ability to transplant solid animal organs is many years away (see page 324), despite claims to the contrary by some biotech-



Allan: 'precautionary principle' must be followed.

This briefing has been written by Declan Butler, European correspondent for *Nature*, with additional reporting by Meredith Wadman in Washington, Sally Lehrman in San Francisco and Quirin Schiermeier in Munich.

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UK ahead in moves to regulate

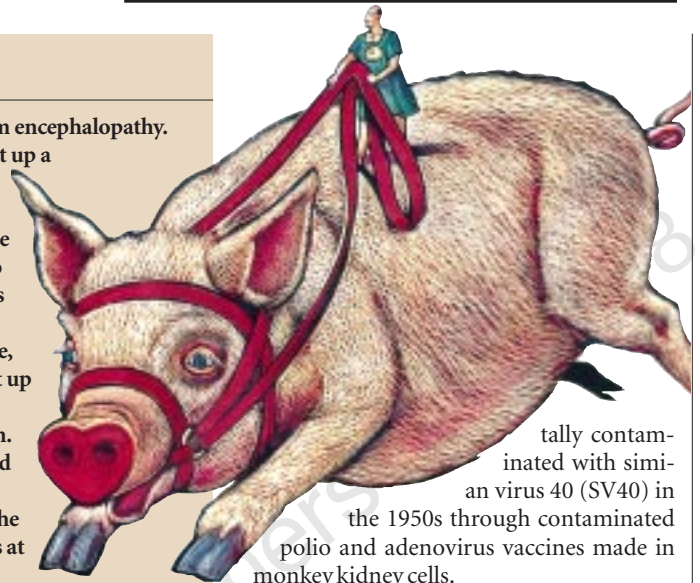
No country has yet judged that the risks of xenotransplantation so outweigh the potential benefits that they justify a permanent ban on clinical trials. Debate is advanced in the United Kingdom and the United States, but is only just beginning in most other countries and at the international level.

The United Kingdom has, for the moment, the most stringent position of any country, having last year introduced a moratorium on clinical trials until further research shows that transplants are safe and that the science is sufficiently developed to offer transplant recipients real benefits (see *Nature* 385, 285; 1997). "The moratorium is useful in that it is allowing the science base to catch up and quantify what is a hypothetical risk," says Ron James, chief executive officer of Protein Pharmaceuticals.

The need for a regulatory framework had been prompted by the announcement in 1995 that Imutran, a Cambridge-based biotechnology company, planned to proceed with clinical trials of pig hearts (see *Nature* 377, 185; 1995), and subsequently sharpened by the political climate following the failure of expert committees to prevent the crisis

over bovine spongiform encephalopathy. The government has set up a Xenotransplantation Interim Regulatory Authority to oversee the field, and is expected to produce legislation this year.

Elsewhere in Europe, Sweden has recently set up a national commission on xenotransplantation. Researchers have agreed to a voluntary moratorium pending the outcome of discussions at the commission which will meet for the first time this month. A similar moratorium is in place in Germany, while France has created an advisory committee within its national transplant authority and intends to release guidelines. France's national bioethics committee is also to review xenotransplantation this year, as is the European Commission's group of advisers on ethical aspects of biotechnology. The commission itself has not taken a position on xenotransplantation.



tally contaminated with simian virus 40 (SV40) in the 1950s through contaminated polio and adenovirus vaccines made in monkey kidney cells.

Weiss points out that the pathogenicity of viruses can change unpredictably when they jump species. SV40 does not seem virulent in infected individuals, but many viruses lying dormant in animals, in particular herpes viruses and retroviruses, can become activated and deadly in humans.

He argues that activation of animal viruses might be favoured under transplant conditions, as these remove many barriers to natural means of infection. Viruses that might not usually infect humans would enjoy intimate cell-cell contact in xenotransplants that might favour both infection and recombination with human viruses, a mechanism known to generate pandemic viruses. "I'm not saying you shouldn't do this [clinical xenotransplantation], I'm asking you, 'have you stopped to think?'" says Weiss.

Shutting the barn door?

The PHS guidelines represent an attempt to find a "middle path," says Pendergast, by allowing trials to proceed despite the scientific uncertainties, while attempting, through monitoring of patients, to contain any risk that emerges.

The science and public health issues are "so complicated" that the FDA must strictly regulate the field, she says. "In our job, we hear a lot of quibbling. Isn't a small trial sufficient, do we really have to run so many tests, does it matter that we haven't validated this assay? We are going to expect and demand the best. The stakes are too high not to."

An important part of FDA oversight will be to encourage trial sponsors to generate better data on the risks, according to Amy Patterson, interim deputy director of the Division of Cellular and Gene Therapies at FDA and a leading authority on xenotransplantation. While everyone agrees on the need for more research, there is little consensus as to whether such studies should be done before further trials, or in parallel with them.

nology companies and a few bravado transplant surgeons.

Solid organs are only the tip of the iceberg of xenotransplantation. Less spectacular implants of animal cells and tissues offer potential benefits to much larger numbers of patients — transplants of pancreatic islet cells could cure diabetes, while implants of neuronal tissue could treat Parkinson's and Huntington's disease, multiple sclerosis and the effects of strokes. Excessive haste in moving to the clinic might create a public backlash that could set the field back years, warns the ASTP.

But the dangers of rushing remain strong. Pressure from patient groups and surgeons to move ahead with trials has coincided with an anti-regulation climate in the United States. "It is hard to say stop," says Jonathan Allan, a virologist at the Southwest Foundation for Biomedical Research in San Antonio, Texas and a member of the FDA advisory subcommittee on xenotransplantation. "I'm not optimistic that they [PHS agencies] will do the right thing, and put public health first."

The Trojan pig

As the FDA subcommittee on xenotransplantation was meeting last month in the United States, hundreds of thousands of chickens were being slaughtered in Hong Kong to try to contain an outbreak of H5N1, a fatal flu virus that previously infected only

chickens and ducks. Although the virus had jumped species, it seems to have infected only people who have come into direct contact with fowl, and does not seem to be contagious (see *Nature* 389, 554; 1997).

If a viral stowaway in a xenotransplant similarly affected only the recipient, it would just be one more of the many infectious complications to which immunosuppressed transplant recipients can succumb. The big unknown is whether animal viruses might spread from recipients to others, and possibly cause pandemics. Natural precedents abound — Ebola and Marburg monkey viruses have caused large disease outbreaks in humans while there is compelling evidence that HIV originated from monkey retroviruses.

Some argue that natural outbreaks merely put the risks of xenotransplantation in a useful perspective, in that animals have transmitted viruses to humans throughout history. Paul Herrling, scientific director of Novartis, says the Hong Kong flu outbreak shows "that the risk from other sources is much greater, and that the added risk in view of the life-saving nature of a successful xenotransplantation might be minimal".

Other researchers are less sanguine. "I take the same data and turn it around, saying 'look, this can happen,'" says virologist Robin Weiss. Indeed, accidents have already occurred: millions of people were acciden-

The message to the FDA from its sub-committee on xenotransplantation is that clinical trials should be done with extreme caution, she says.

Paul Noguchi, director of the FDA's Division of Cellular and Gene Therapies, says: "We are committed to examining all the studies very critically and should anything happen we are prepared to halt activities."

The discovery that pig retroviruses can infect human cells *in vitro* has sent researchers scurrying to see whether patients who have already received porcine transplants show signs of infection. "It behoves everyone to 'stop for a moment' and evaluate those people that have already been transplanted," says Marian Michaels, a specialist on risks of xenotransplantation at the Children's Hospital of Pittsburgh.

Novartis is studying 150 patients who have had pig skin transplants for serious burns, implants of pancreatic islet cells, or who temporarily underwent perfusion using pig liver 'bridges' while awaiting a human liver.

In Sweden, studies are tracking ten renal diabetic patients who received fetal pig islets

in the early 1990s, and two patients who had outside perfusions using pig kidneys. Twenty-four neural tissue recipients are being tracked in Boston, Massachusetts. Ann Tibell, a researcher at the Karolinska Institute in Stockholm who is leading the pig islet study, recently reported antibodies reactive to swine influenza in all ten patients, porcine parvovirus in five, and five other pig viruses. Only one of the patients is ill, with parvovirus. Tibell is checking whether the results may be accounted for by cross-reactivity with human viruses, and retrovirus assays have yet to be completed.

"As a result of these studies we will have much more data [on risk] in a year from now," says Louisa Chapman, from CDC. But Jonathan Allan, a virologist who sits on the FDA's advisory subcommittee on xenotransplantation, thinks the agency is failing to pay sufficient attention to the 'precautionary principle'. "Xenotransplant researchers haven't done their homework," he says.

Allan is particularly concerned about the threat that, were a xenozoonose to behave like HIV or HTLV, it could spread quietly for

decades before being detected, by which time it might also have contaminated the blood supply. The FDA is attempting to address such concerns by requiring monitoring of all future xenotransplant recipients for infectious diseases over their entire lifetime, and prohibiting them and their close contacts from donating blood.

Some argue that this approach could amount to shutting the barn door after the horse has bolted. But this interpretation is disputed by André La-Prairie, official at Health Canada, the country's equivalent of the FDA, which is drafting guidelines similar to those of the United States. He admits that monitoring may not be a guarantee against infections escaping into the population, but argues that "it should tell you as soon as possible if and when the horse has left the barn," and allow trials to be quickly stopped.

Similarly, virologist David Onions points out that what is under discussion is experimental clinical trials, not therapeutic programmes involving thousands of recipients. "Five to ten people you know about are not going to start an epidemic," he says.

But Allan and others argue that the proposed monitoring measures are burdensome, and that their implementation is likely to be unworkable and vulnerable to human errors. "If you are putting your bets on containment, it's a lost cause," he asserts.

Indeed, the draconian monitoring measures being proposed are unprecedented in medicine. They would transform the nature of informed consent into a 'binding contractual agreement'. Patients would surrender the traditional right to withdraw from experimental procedures at any time, and be obliged to accept lifetime monitoring.

Recipients might even face quarantine, in much the same way as astronauts returning from the Moon were initially isolated for several months in case they brought back weird diseases, according to Abdullah Daar, a transplant surgeon at the Sultan Quaboos University in the Sultanate of Oman, who is an authority on the ethics of transplantation and chairman of the xenotransplantation advisory committee set up last year by the World Health Organization.

Trials jump the gun on science

The stringent precautions that xenotransplantation seems likely to require, and the prospect that it may never be possible to eliminate its potential risk to the public, raises the question of whether the risks would be outweighed by the benefits. There is a broad consensus that eventually, if xenotransplantation proves technically feasible, the answer will be 'yes'. There is less agreement on whether the science is sufficiently advanced to justify moving to clinical trials now.

Optimism that clinical applications might be near rose in 1995 when Imutran announced that it had overcome one of the

Confronting the risks of 'xeno-havens'

The announcement last year of the cloning of Dolly the lamb led to an international response unprecedented in medical ethics. Within hours, Presidents Clinton, Chirac and Santer had called on their ethics committees for advice on the implications for humans, while international agreements on bioethics that had been all but finalized by the Council of Europe and Unesco were quickly modified to include a ban on human cloning for reproductive purposes.

"The wrong issue for a moral panic," sighed at the time David Shapiro, then executive director of Britain's Nuffield Council on Bioethics, arguing that if there was an area of medical ethics in dire need of international regulation it was not cloning but xenotransplantation. For, however hard countries such as the United States or United Kingdom attempt to regulate xenotransplantation to reduce the risk of creating human diseases, their efforts will be in vain if other countries adopt weaker or no regulations. Such countries could become 'xeno-havens' for unscrupulous surgeons, allowing animal viruses to escape into the human population (*Nature* 385, 378; 1997).

"The prospect scares the hell out of me," says Daniel Salomon, a scientist at Scripps Research Institute in California, and member of the board of the American Society of Transplant Physicians, a body that has long waged war on the trafficking of human organs in developing countries. The exorbitant costs of meeting the stringent regulatory requirements for clinical xenotransplantation now being developed in

the United States and other industrialized countries will create a strong incentive for traffic in xenotransplants with 'xeno-havens' providing cheaper alternatives, argues Salomon. The big risk, he says, is that humans will end up with virus-laden organs from baboons and chimpanzees, rather than from specially bred pigs which — even given the acknowledged dangers — should nevertheless be safer.

The European Commission has yet to take a position on clinical applications of xenotransplantation. Ironically, one reason is that the agenda of the scientific steering committee that advises the commission on technical issues is currently jammed with issues arising from the aftermath of the bovine spongiform encephalopathy crisis, according to Paul Vossen, a commission spokesman.

One official from the World Health Organization (WHO) admits that the growth of interest in xenotransplantation has caught the organization off guard. It has since moved speedily, setting up a xenotransplantation advisory committee last autumn, and planning to release guidelines on the technology within the next few months.

But many argue that WHO guidelines are not enough, pointing out that WHO is not a regulatory authority, so any regulation of xenotransplantation technology at the national or international level will be up to national governments. "We need some kind of formal binding international agreement," says Salomon.

major barriers of cross-species rejection, hyperacute rejection (HAR), a violent immune response involving the complement system that is unleashed when organs are transplanted between distant relatives such as pigs and humans. HAR destroys the blood vessels in the organ, cutting off oxygen, and killing it within minutes.

Imutran's announcement followed experiments in which the company claimed cynomolgus monkeys given genetically engineered pig hearts heterotopically survived for as long as 60 days, with none of the hearts suffering from HAR, whereas control animals died within one hour of transplantation. David White, the company's medical director, said the technology was now "ready for testing in humans" (see *Nature* 377, 185; 1995).

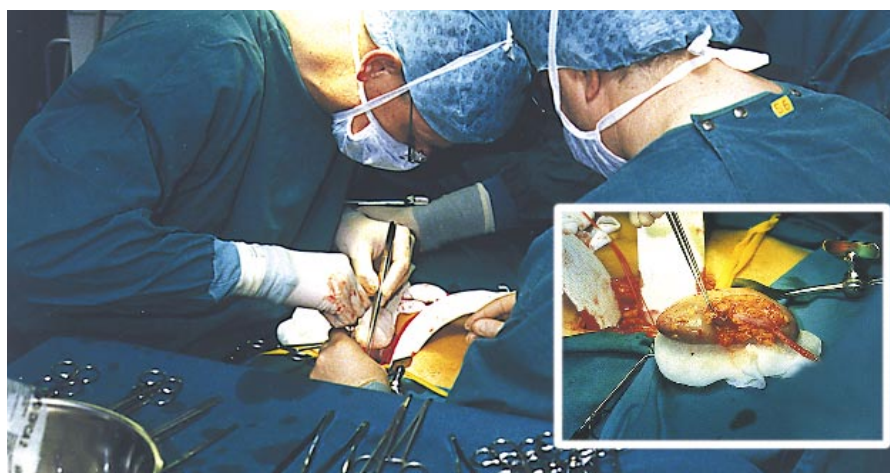
At least three other companies have also engineered pigs to overcome HAR: Protein Pharmaceuticals, Alexion of New Haven, Connecticut, which is linked to US Surgical Corporation, and Nextran of Princeton, New Jersey, a subsidiary of Baxter. But there is now a growing awareness that, even if HAR is overcome, this alone is unlikely to be sufficient to prevent rejection of organs, admits Paul Herrling, research director of Novartis, which bought Imutran in 1996.

Delayed xenograft rejection, which occurs within days when macrophages and natural killer cells invade the organ, is widely considered as formidable a barrier as HAR. And, although the remaining major barrier, the T-cell response, should in principle be able to be controlled using conventional immunosuppressors, recent research suggests the T-cell response to xenografts is subtly different and may require the development of new immunosuppressors.

Herrling rejects White's optimistic projections as premature, and says the company has no immediate plans for clinical trials of transgenic hearts, because it feels that not all the "physiological and pharmacological issues have been satisfactorily solved". He says Novartis is investing heavily to try to "develop clinically acceptable immunosuppressive regimes," and that this is likely to require new approaches to those used in allotransplantation — transplants using human organs, cells or tissues.

Hype hides obstacles

Some accuse capital-hungry biotechnology companies of generating excessive 'hype' about the prospects of xenotransplantation and of having created unrealistic expectations among patients, fuelling pressure to proceed to clinical trials. The ASTP warns that the domination of the research by companies "may not be in the best interests of the community," arguing that "early clinical trials may be sought without adequate documentation or publication of support data in order to facilitate corporate interests".



Kidney transplant: a victim of its own success.

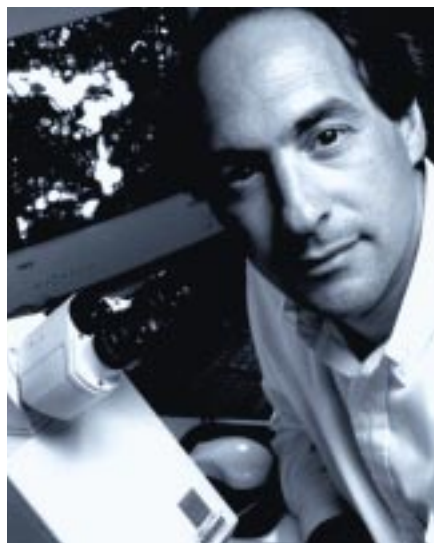
Herrling says excessive hype has become "much less of a problem since the acquisition of Imutran by Novartis". The buyout means that Imutran has "no short-term financial pressure to rush into clinical trial prematurely as might be the case with a standalone biotech company dependent on shorter-term financial goodwill".

Need for novel approaches

In the cold dawn of the 4th International Conference on Xenotransplantation last autumn in Nantes, France, it was clear that optimism resulting from progress in overcoming HAR had given way to the realization that many other factors playing a role in organ rejection are still far from understood.

Analysts are similarly sceptical about the short-term prospects for clinical success. "The immune system is unbelievably complicated and poorly understood, making xenotransplantation one of the most speculative of all areas of biotechnology," says Alex Zissson, an analyst at Hambrecht and Quist in New York. "Companies are years away from having a product on the market."

Daniel Salomon, a scientist at Scripps



Salomon: 'science base still insufficient'.

Research Institute in California and member of the board of the American Association of Transplant Physicians, says he believes the risks of xenotransplantation are "manageable," but feels that the current lack of scientific understanding of rejection and organ function precludes clinical application for some time to come. He thinks a case can be made for trials with the minimum number of patients needed if this yields information that can "move the field forward," however.

The holy grail of allotransplantation, completely avoiding the problem of rejection by re-educating the recipient into recognizing donor tissues as 'self', is being pursued by the team of David Sachs, a professor of surgery at Harvard Medical School and director of the transplantation biology research centre at Massachusetts General Hospital, in cooperation with Bio-Transplant, a company linked to Novartis. Applied to xenotransplantation, this technique would similarly overcome the T-cell response, but would itself be insufficient to overcome cross-species rejection and would need to be used with techniques to overcome hyperacute and delayed xenograft rejection.

The concept is elegantly simple. Bone marrow cells from the donor are first engrafted into recipients, while at the same time temporarily disabling their immune system; as the system recovers, the recipient develops tolerance to the organs and tissues of that donor. Sachs points out that this would, in principle, avoid the lifelong immunosuppression currently needed by transplant patients.

In a major breakthrough, Sachs's group reported last year that they had induced permanent tolerance pig skin grafts in mice using this technique (see *Nature Medicine* 2, 1185 & 1211; 1996). Stephen Squinto, vice-president of research at biotechnology company Alexion, admits that the technique is "scientifically interesting". But he points out that its clinical applicability will require a more gentle technique for disabling the immune system than that used by the group

Primate risks 'still going unheeded'

One controversial aspect of the proposed US guidelines on xenotransplantation is the lack of an explicit ban on the use of organs from non-human primates, such as baboons.

Although these are less susceptible to rejection because of their close similarity to human organs, they are nonetheless widely considered unsuitable for transplantation because of their much higher perceived disease risk, and the fact that it would be impractical to breed the large numbers of 'clean' animals that would be needed.

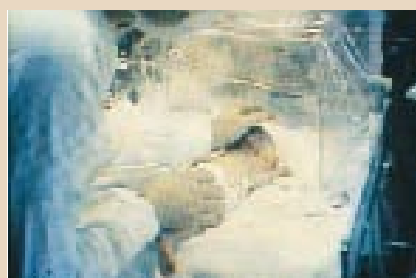
Such considerations led an ethics panel set up by the UK government to rule out the use of primates as donors on the grounds that pig pathogens are better characterized and the animals are easier to breed in large numbers under clean conditions.

But many scientists are unhappy about the lack of a US ban on the use of primates, given their unsuitability. US agencies appear to have felt that the issue was not the species used, but rather the level of disease risk. Applications for clinical trials would be judged on the basis of how well-defined the pathogens of a particular species were, how easily they could be removed, and on the risks that they harboured unknown viruses, says Louisa Chapman, an official at the US Centers for Disease Control and Prevention. She argues that in practice non-human primates will have much greater difficulty in meeting these criteria than pigs.

Such assurances are met with scepticism by critics who point out that the US Food and Drug Administration (FDA) approved a controversial trial of baboon bone marrow in AIDS patient Jeff Getty in 1995 (see *Nature* 378, 756; 1995).

Suzanne Ildstad, director of the Institute for Cellular Therapeutics at Allegheny University of the Health Sciences in Philadelphia, who oversaw this trial, is keen to continue with further trials.

At least one other surgeon also has plans to transplant solid organs from non-human primates. Leonard Bailey, from the Loma Linda University Medical Center in California, one of the country's top heart



Baby Fae: despite failure, new operations with baboon hearts are still being planned.

transplant surgeons, says he intends to apply to the FDA to transplant hearts from the centre's baboon colony into children. "We don't want to risk the public health, but we don't think we need to hold back on the basis of speculation about risks to public health."

In 1984, Bailey carried out the most celebrated xenotransplant operation, placing a baboon heart into a two-week old baby — Baby Fae. The child died three weeks later after her immune system destroyed the organ. Bailey now claims to have obtained "prolonged survival" in animal studies (see *World Journal of Surgery* 21, 943–950; 1997) and intends to try again.

But another surgeon who also pioneered early baboon xenotransplants, Thomas Starzl, from the University of Pittsburgh, says he has decided not to proceed for the time being. Starzl carried out a series of unsuccessful baboon-to-human kidney transplants in the early 1960s and again in the 1990s. But he says lack of scientific understanding means that "we are too far from being able to do anything [clinically]; we are tremendously interested but we think the research endeavours are going in the wrong direction".

Concern about the use of non-human primates has been heightened by a loophole in the guidelines that would seem to risk allowing the use of virus-laden wild primates. The revised guidelines do not explicitly ban the use of these, saying only that departures from ideal husbandry would need to be justified by the trial sponsor.

— whole-body irradiation, thymectomy and purging the body of T-cells.

cine 3, 978–983; 1997), in which bovine adrenocortical cells implanted into immunodeficient *scid* mice were able to develop into functional adrenal tissue in the kidneys of mice from which the adrenal glands had been removed. This suggests that the only absolute barrier to wider use of such implants is the immune system.

Clinical trials are already under way worldwide. Ole Isacson's team at Harvard Medical School, for example, are transplanting patients with immortalized mouse fibroblasts, producing retrovirus vectors to deliver a therapeutic gene to brain tumours,

and with fetal pig neurons to try to replace dopaminergic neurons destroyed in Parkinson's disease (*Nature Medicine* 3, 964; 1997).

The fact that cells and tissues seem closer to clinical success means that trials involving these are much more likely to be approved than ones involving solid organs, predict US regulatory officials. Indeed, even if rejection can be overcome, maintaining the complex functioning of solid organs in a human host "for a sufficient time to make clinical sense," remains a major challenge, says Herrling.

Call for moratorium

As the potential risks of xenotransplantation would affect the general population were they to materialize, approving trials through the traditional regulatory approach could be interpreted from an ethical standpoint as tantamount to exposing the public to these risks "without their consent or awareness," according to Bob Arnold, from the Center for Medical Ethics at the University of Pittsburgh. He poses the question of whether the public should be given a direct say in weighing up the risks and benefits of the technology, while at the same time noting the difficulty of defining "what sorts of public action would constitute consent".

Although the US PHS solicited broad public comments on its initial guidelines, the subsequent decision-making process itself has been restricted to within the regulatory agencies. "It is odd that a small number of people in the federal government are making unilateral decisions about something that could have such long-term consequences for the public," says Allan. He points out that expert committees have not been infallible in their handling of such issues where the risks are remote but the public health consequences potentially serious, as has been



Bach: calling for a xenotransplant scientist moratorium.

amply shown by the bovine spongiform encephalopathy (BSE) crisis, and the contamination of blood supplies with HIV during the 1980s.

Fritz Bach, a leading xenotransplant scientist from Harvard Medical School, Boston and a proponent of continuing basic research in this area, argues that, before expert committees issue regulations on clinical trials, there should be a wide "informed" public debate on the question of whether such trials should be allowed to proceed at all at present (see *Nature Medicine* 4, 142–145; 1998). The question is ultimately an ethical, and not a technical, one, says Bach: "Is the risk to the public, which we can't quantify but which we know is greater than zero, justified by the help we are going to give individuals?" The FDA is "neutral" on the question of whether a moratorium is needed, says Noguchi.

Closer to the clinic?

Cells and tissue xenotransplants are less vulnerable to hyperacute rejection than organs because they have no blood vessels for HAR to attack, relying instead on the supply of blood from the host, although they must overcome a strong cellular response.

The potential for such implants looks promising, as shown by the recent report by Michael Thomas, from Baylor College of Medicine at Houston, Texas (*Nature Medi-*

Alternative ways of meeting demand

The heated debate on both the potential benefits and dangers of xenotransplantation threatens at times to overshadow the fact that the use of animal organs is not the only possible response to the chronic shortfall between the demand for human organs and the supply.

The growing waiting list for human organs in the United States (see figure) mirrors the upward trend worldwide. The number of transplant patients is relatively small in public health terms, compared with say the 500 million cases of malaria worldwide. But the costs of individual operations are such that transplants constitute a billion-dollar market, which in turn is fuelling intense commercial interest in xenotransplantation.

Demographic trends suggest that the demand for organ transplants is set to increase further, particularly as many of the diseases that result in organ failure — such as renal failure caused by diabetes — are age-related. Meanwhile, a decline in traffic accidents due to improved safety is resulting in a drop in the most reliable source of organ donors, accident victims.

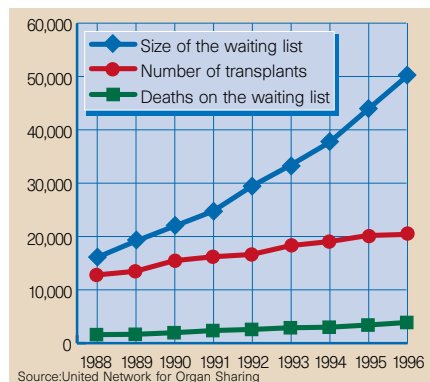
Transplant statistics alone give a misleading impression of the scale of the problem, as many more individuals might seek treatment if an unlimited supply of organs were available. David Sachs, of Harvard Medical School, estimates that more than 400,000 people in the United States could benefit from heart transplants; yet the official waiting list for hearts in 1996 was just 3,698.

Xenotransplantation is not the only means of alleviating this shortage. Boosting the low rates of cadaveric donations — which vary from 27 per million inhabitants in Spain and 21 in the United States to 5 in Greece — would go some way towards this goal. Countries such as Austria, which have 'presumed consent' laws that do not require next of kin to be notified or to give their consent to organ donations from the newly dead, generally have higher donation rates than countries such as the United States where informed consent is required.

Donation rates also seem to be highly sensitive to public attitudes. Transplant centres often experience sharp drops in donations following media coverage of such events as the kidnapping of children for organs or the use of organs from executed prisoners.

Shortly before Christmas, US Vice-President Al Gore and Donna Shalala, Secretary of Health and Human Services, launched a campaign to increase donation rates.

Policy changes, such as encouraging pre-



The growing US waiting list for human organs is fuelling demand for transplants of animal organs.

vention of heart disease or encouraging more donations, may go some way to alleviating the shortage of organs. But there is a broad consensus that these will not be sufficient, and that only science can ultimately improve the situation.

Xenotransplantation, if it proves feasible and safe, seems to offer a panacea. Apart from the risks of spreading animal diseases to the human population, it would otherwise bring a quality control to transplantation that is currently lacking, as organs could in principle be prepared in advance in large numbers, individually screened for infectious disease agents.

Alternative research routes

But other research avenues may also have an impact on the organ shortage. One is to improve the success rate of transplant operations, as many of those on the waiting lists are for repeat procedures to replace failed transplants. Although survival rates of allotransplants have been greatly improved by the introduction of the immunosuppressant cyclosporin, chronic rejection remains a major cause of loss of allotransplants, for example, according to Paul Herrling, research director of Novartis. The company has 200 scientists working on this problem.

Artificial organs and human cells and tissues provide other alternatives to xenotransplantation, and would avoid the risk of xenozoonoses. Novartis is about to market an artificial skin, Apligraf, based on cultured human cells, for example, while stem cells extracted from umbilical cord blood offer a promising alternative to bone marrow transplants (see *Nature* 382, 99; 1996).

Herrling says Novartis only intends to use xenotransplants where no alternatives are currently available, such as for hearts and liv-

ers. Similarly, the company only intends to proceed with clinical trials in the first instance for life-saving indications. It would not, for example, test kidneys where alternatives such as dialysis exist, he says.

Research to create next generation artificial hearts is also under way, although reliable devices are still some way off. More complex organs, such as artificial livers and lungs, remain even more distant possibilities. Another promising alternative in the long term, in particular for cell and tissue implants, would be to graft tissues derived from human embryonic stem (ES) cells. In principle these could be cultured to provide large quantities of a range of differentiated tissues such as nervous tissue or muscle.

The therapeutic potential of such cells led the French national bioethics committee to recommend last year that a ban on human embryo research be lifted for research in this area. But the culture of human ES cells has so far proved impossible. Even the culture of animal ES cells — although more straightforward — is still in its infancy.

ES cells also have a potential drawback in that they may give rise to cancers. But interest in their transplant potential was nonetheless raised last summer, when John Gearhart, a researcher at the Johns Hopkins University School of Medicine in Baltimore, told the International Congress of Developmental Biology in Snowbird, Utah, that his group had achieved growth of human ES stem cells *in vitro* for several months.

Ronald McKay, head of the laboratory of molecular biology at the US National Institute of Neurological Disorders and Stroke, is using ES cells in a rat model to generate large quantities of dopaminergic neurons to treat Parkinson's disease. He believes that, in the long term, culture of human ES cells will have a major impact on transplant biology.

"I think we will be able to graft cells, and if one knows enough about kinetics and control mechanisms of cellular differentiation then you will be able to restore organs to health," he predicts. "It might sound like science fiction but it seems plausible; reconstructing organs is a natural extension of developmental biology."

McKay argues that transplantation is still in the "hunter-gatherer" phase, and that the way ahead is the "settled agricultural" phase, namely establishing banks of well characterized cells with strict quality control. Animal experiments suggest that ES cells could be engineered to be accepted by the immune system and to generate 'universal donor' tissues acceptable to all patients.

One attraction of such cells is that only a few embryos would be needed to seed banks of cells without needing new abortion material. But several US scientists argue that the strength of political opposition to abortion in the United States means that such research is unlikely to be encouraged. □

Call for moratorium on xenotransplants

Sir — The Asilomar moratorium on applications of recombinant DNA research, agreed to by molecular biologists in 1974, marked a turning point in the approach of biologists to their responsibilities to the public in developing a technology with unpredictable consequences.

That the worst-case scenarios envisaged at the time did not materialize in no way detracts from the merit of the caution taken. Today, we are once again faced with a similarly perplexing quandary.

Xenotransplantation, the transplantation of animal organs, tissues and cells, promises substantial benefits in the long term^{1,2} yet also creates a risk that infectious agents from the donor animal might jump the species barrier to man, not just infecting transplant recipients but also spreading to the general population³. We

believe that a decision on whether to proceed at present with clinical trials of xenotransplantation should not be left to the traditional technical-based approaches that regulatory agencies use to evaluate new medical technologies.

Given the potential risk to the public, the issue is first and foremost an ethical one. Before introducing a regulatory framework driven by technical considerations, an informed public debate is needed so that the public can decide whether it wishes to consent to clinical xenotransplantation at all and, if so, under what conditions.

Until such a review is completed in the United States, we advocate a moratorium on all forms of clinical xenotransplantation, a recommendation discussed more fully elsewhere⁴. At the same time, fundamental research in xenotransplantation should be

actively supported, given that it promises not only to advance our understanding of the immune and vascular systems, but also to fill some of the many gaps in our understanding of the problems, benefits and risks of potential clinical application of this technology.

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Equal opportunities in Canada

Sir — Prompted by the report of a large difference between award rates for women and men in the postdoctoral fellowships competition of the Swedish Medical Research Council (*Nature* 387, 341; 1997), the Medical Research Council of Canada has assembled data on approval rates in its own programmes.

MRC-Canada expects that women and men should have equal opportunity in competitions for grants and awards. To that end, we advise members of selection committees that, when assessing the scientific achievements of applicants, they should take into account factors such as time devoted to child-bearing and raising.

For the Operating Grants programme, MRC-Canada's principal mechanism for supporting high-quality research projects, the approval rate over a three-year period was 25.4% for applications led by women (297 out of 1,167) and 26.6% for applications led by men (1,160/4,368). A Chi-square test reveals that the difference is not statistically significant.

In competitions for MRC-Canada scholarships, an award that provides five years of salary support for recently trained researchers, there is similarly no statistically significant difference between the approval rates for applications from women (14% or 20/143) and men (16.6% or 64/386).

MRC-Canada fellowships offer personal support for two types of developing researcher: PhD graduates who are pursuing postdoctoral training and health professionals who are undertaking intensive

training in research. For five fellowship competitions, the overall approval rate is 12.9% for applications from women (111/858) and 16.3% for applications from men (222/1,361).

The difference is statistically significant ($p < 0.05$). Nevertheless, in two of the five competitions, the approval rates for applications from women and men are virtually identical.

We should expect to find variation in scientific review processes, not only among agencies around the world, but also among programmes. For example, at MRC-Canada we use classic peer review in the assessment of proposals for operating grants whereas for fellowships we ask a multidisciplinary committee to assess the research potential of candidates. It is reassuring that award rates in operating grants competitions indicate equal opportunity for women and men scientists. For research training programmes, such as fellowships, where candidates are not yet established scientists, we believe that assessment criteria should include not only the candidate's research accomplishments and projects (both of which may have been strongly influenced by past and present research supervisors) but also the candidate's critical ability, independence, perseverance and so forth.

We are working to ensure that none of the criteria involved is systematically influenced by gender.

Henry G. Friesen

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Kuhnian pastiche

Sir — Your leading article of 8 January¹ worries about the apparent disregard of biology for Thomas Kuhn's ideas, and seeks an explanation for biology's growing public profile. The latter is an interesting problem for the sociology of science and deserves attention, the former is an empty issue. Might it not be that biology does not fit in simply because of major limitations in the Kuhnian conceptual apparatus itself?

Margaret Masterman² once found that, within the confines of his short book, Kuhn had used the term 'paradigm' with about 21 different meanings. Equally obscure is the mechanism called 'Kuhnian revolution', and the 'incommensurateness' version will not do as it begs more questions than it answers. For how is it to be adjudicated upon? If an encompassing frame or universal perspective did already exist to ascertain true 'no derivation' of one paradigm from the other, why not use this very external frame to describe and explain scientific change?

Kuhn's ideas were a bad pastiche of Gaston Bachelard's. The great Frenchman kept well away from facile accounts, particularly from those that 'solved' the problem in abstract and had no regard for the history of the specific sciences.

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Transgenic pigs and virus adaptation

Pigs offer the best hope of providing organs for transplantation to humans. But, in overcoming the problems of tissue rejection, we may be increasing our risk of infection from pig viruses.

Robin A. Weiss

Homer's story of what Circe achieved with an early formulation like cyclosporine has inspired the name of a biotechnology company that provides pig hepatocytes for *ex vivo* treatment of fulminant liver failure, and we named a pig retrovirus in her honour. Down the ages there have been many tales of men being transformed into beasts, and princes into frogs. Marie Darrieussecq's *Truismes*, now available in English translation¹, is a modern parable on the porcine fate of a young woman in a 'massage' parlour who gradually turns plumper and pinker and who begins to grunt and squeal in a way that initially delights her clients. Billed as a feminist version of Kafka's *Metamorphosis*, it probably owes as much in its undertones of fascism to Ionesco's *Rhinoceros*. Conversely, anthropomorphism in animals is an equally popular genre for cautionary tales, ranging from Aesop's *Fables* to the telling climax in Orwell's *Animal Farm* when the pigs totter around on their hind legs. It is this second aspect, the humanization of pigs, that I wish to address in this commentary on xenotransplantation — in which tissue is transferred from one species to another — and infection.

Preventing hyperacute rejection

Monkeys are unlikely to provide an adequate supply of clean tissues for xenotransplantation, so most investment is being made in the use of pigs². Although there has been limited success in transplanting pig islet and neural cells, a major barrier to the 'discordant' xenotransplantation of whole organs from pigs into primates is a phenomenon known as hyperacute rejection. This involves the destruction of the vascular endothelium of the donor organ within minutes of exposure to human or monkey blood.

The lysis of endothelial cells occurs through a complement-dependent mechanism following the binding of naturally occurring antibodies to carbohydrate antigens on the glycolipids and glycoproteins of the donor cells. It is akin to the destruction of red blood cells in ABO-mismatched transfusion. The main xeno-antigen recognized on porcine endothelial cells is a galactose $\alpha(1-3)$ -galactose terminal sugar residue (α Gal)³. Pigs and most mammals express an $\alpha 1-3$ galactosyltransferase, which places α Gal on glycoconjugates. Along with other old-world primates, humans lack this enzyme; we appear to be natural genetic knockouts for the gene encoding galactosyltransferase. Because we are exposed to α Gal antigen (for example, on



Circe offered Odysseus's men a potion mixed with cheese, honey and wine. And when they had emptied their bowls, they grew pigs' heads and bristles, and they grunted like pigs; but their minds were as human as ever.

bacteria in the gut) we respond by making antibodies to α Gal. Indeed, more than 2 per cent of total human IgM and IgG in the circulation represents α Gal antibody⁴, and this triggers the complement-mediated lysis of xenogeneic cells bearing α Gal antigens.

Several strategies are being developed by biotechnology companies to block hyperacute rejection and so help porcine tissues to survive in humans^{2,5}. One approach involves circulating human blood plasma over α Gal-immunoadsorbent columns to remove anti- α Gal antibodies; unfortunately, anti- α Gal levels quickly return to normal in the blood. Another method is to inhibit triggering of the complement cascade by treatment with antibodies to complement components or with excess amounts of a soluble form of complement receptor. A third strategy is to generate transgenic pigs that overexpress a fucosyltransferase that competes with the substrate for α Gal attachment so that a fucose group, representing a natural human blood-group antigen, is substituted for α Gal. A fourth idea is to knock out the pig gene that encodes $\alpha 1-3$ galactosyltransferase. This would eliminate the main cause of hyperacute rejection in the first place, but the technology for porcine knockouts is not yet available. The fifth strategy is to generate transgenic pigs that express human comple-

ment-regulatory proteins. These proteins, CD55 (also known as decay accelerating factor, DAF), CD46 (membrane cofactor protein, MCP-1) and CD59 (protectin) do not prevent anti- α Gal antibody binding to endothelial cells to trigger the classical pathway of complement activation; rather, they inhibit downstream steps in the complement cascade so that cell lysis may be prevented. Several transgenic pig herds have been developed that express one or more of the human genes encoding CD55, CD46 and CD59.

Virolysis

Many animal viruses with lipid envelopes are sensitive to inactivation by human complement. Virolysis was first observed with retroviruses⁶. It was thought to explain in part why we seldom if ever become infected with the retroviruses frequently shed by our animal neighbours, such as cats and mice, even though some of these viruses readily replicate in human cells in culture. At first it seemed that lysis of animal retroviruses by complement occurred without specific antibody, because all human sera showed this activity. But we now know that lysis of retroviruses is triggered by the binding of anti- α Gal antibodies in human sera to α Gal residues expressed on the viral envelope^{7,8}.

Virus grown in non-primate cells is sensi-

tive to inactivation by fresh human serum, whereas the same virus propagated in human cells is not. Other enveloped viruses grown in animal cells are also sensitive to lysis by human complement, including arenavirus, paramyxovirus, alphavirus and the rhabdoviral pig pathogen, vesicular stomatitis virus^{8,9}. If α Gal is on the host cell then the viral envelope becomes sensitive to rapid lysis by human serum. In other words, virus inactivation occurs by precisely the same mechanism as hyperacute rejection of xenografts.

It follows that modifications to make porcine xenografts resistant to hyperacute rejection may also make any enveloped viruses of pigs similarly resistant to lysis. This must be so for the removal of anti- α Gal antibodies from the patient's plasma, or for the removal of α Gal from the surface of pig cells by competing fucosyltransferase or by knocking out galactosyltransferase function. It might also be the case where pigs are transgenic for human complement-regulatory genes, provided that enough of the human proteins are incorporated into the viral envelope.

Proteins CD46, CD55 and CD59 are present in the HIV envelope^{10–12} and can protect HIV from complement-mediated lysis^{10,11}. Whether the presence of human complement regulatory factors in pig cells changes the serum sensitivity of viruses budding from them still needs to be rigorously tested. But pig endogenous retrovirus released from pig cells is highly sensitive to human complement-mediated lysis, whereas the same virus passaged in human cells lacking α Gal immediately becomes resistant¹³.

The extent to which anti- α Gal and complement protect us from infection by animal viruses is unknown. Individuals with deficiencies in the complement pathway tend to be more susceptible to bacterial than viral infections. But the possibility remains that viral zoonoses are more likely to occur in xenotransplantation of transgenic rather than unmodified pig tissue.

Human receptors in transgenic pigs

Another, neglected facet of porcine transgenesis aimed at blocking hyperacute rejection potentially adds considerably to the risk of cross-species infection. Two of the three human complement regulatory proteins are also receptors for human viral pathogens: CD46 is the cell-surface receptor for measles virus¹⁴, and CD55 can serve as a binding receptor for Echo and Coxsackie B picornaviruses^{15,16}. Coxsackie B virus causes myocarditis and might endanger the pig heart in an immunosuppressed recipient of a xenograft. Clearly, valuable transgenic herds should not be husbanded by infected staff in case the pigs become infected by measles virus or picornaviruses. But the converse situation worries me more.

Transgenic pigs may provide an opportunity for animal viruses to adapt to a human

host range. Human Coxsackie B virus, for example, can be adapted to grow in mice, and in some human cell cultures it increases its infectivity a million-fold by adopting the CD55 receptor¹⁶. If pigs were to harbour picornaviruses that use the porcine equivalent of CD55, such viruses might readily adapt to recognize human CD55 in transgenic pigs that express both the native porcine and the human forms of the receptor. These viruses would then be preadapted for transmission to the xenograft recipient and for human-to-human transmission. There is already concern that mice transgenic for the human poliovirus receptor should not escape and become a non-human reservoir for a human pathogen.

Animal morbilliviruses (measles-related viruses such as canine distemper virus and rinderpest virus), too, might become preadapted for human transmission in CD46-transgenic pigs. Morbilliviruses are known to jump host species to wreak havoc, as in the recent epidemic in seals and dolphins. In Australia a veterinarian and a stable-hand died after an autopsy of a horse with a new type of morbillivirus infection, which in turn was probably acquired from fruit bats¹⁷. However remote, we should be mindful of the additional public health risk of genetically modified pigs that express human virus receptors.

Balancing benefit and risk

It is sometimes said that because pigs and humans have lived together for thousands of years there cannot be new microbes to cross between these species. Notwithstanding pigs as a source of new influenza pandemics, xenotransplantation will potentiate the risk of pig-to-human transfer of viruses not transmitted by the respiratory route. First, the physical barrier is broken by transplanting living porcine tissues or organs into humans. Second, the immunosuppression required to prevent xenograft rejection may allow zoonotic viruses to adapt to human infection. Third, as discussed above, the genetic modification of pigs may allow preadaptation of animal viruses for human infection.

None of these risks is easily quantifiable. We know that human tumour tissue xenotransplanted into immunodeficient mice frequently becomes infected by endogenous, xenotropic mouse retrovirus. Thus pig-to-human viral transfer could also occur by xenografts, as we have found that two of three distinct porcine retroviruses can infect some human cells in culture^{13,18}. Moreover, because these viruses are endogenous (that is, their genomes are carried as Mendelian traits in the pig chromosomes), raising virus-free pigs will not be easy. Horizontally transmitted viruses may be more readily excluded through screening, provided that we know of their existence. But we may not know; only a few months ago, a new porcine virus related to human hepatitis virus E was reported¹⁹. How many more

potential pathogens remain to be discovered?

To the individual xenograft recipient, the benefit of a successful transplant will almost certainly outweigh the risk of any subsequent untoward effects of infection by a pig virus. The transplant surgeon can therefore sleep with a clear conscience that he is helping his patient. We microbiologists, on the other hand, wish to alert society to the more remote but possible risk of setting off a new human epidemic. After all, HIV-1 probably began by cross-species transfer (HIV-2 certainly did). I would not expect the pig endogenous retroviruses to present such a problem, because they grow to only low titres in human cells and so are unlikely to be pathogenic. But we cannot dismiss virus adaptation or recombination with other retroviruses in the new host.

We need a Hippocratic ethic for community and public health, at least to do no harm. Fred Murphy²⁰ remarked that the acceptability of xenotransplantation at the level of social risk has followed a rollercoaster course. Personally, I am in favour of moving forward — cautiously — neither prohibiting human xenotransplantation nor rushing headlong like the Gadarene swine over this precipice of new technology.

We need to set in place the appropriate system to monitor possible virus transmission in human xenotransplant trials. Before proceeding, however, we should find out as much as possible about whether xenogeneic infection has occurred in individuals already exposed to live porcine tissue. No one wishes to promote an unforeseen Odyssey of pig viruses journeying through a xenograft recipient to his or her contacts and onwards to the population at large. □

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News of chews: the optimization of mastication

R. McNeill Alexander

In chewing food, we break it into small fragments that are mixed with saliva to form a soft ball, or 'bolus'. A new study reveals that the number of chews is optimized for particular foods — too few, and the bolus doesn't stick together; too many, and it begins to fall apart.

Chewing breaks food down into small fragments, increasing the surface area that is exposed to digestive enzymes. A current view is that we should continue to chew each mouthful until the fragments are small enough (however small that may be), and until they are well mixed with the saliva that will lubricate their passage down the gullet¹. But in *Proceedings of the Royal Society*, Prinz and Lucas² emphasize another function of chewing. They conclude that we should continue to chew until the food fragments bind together as a coherent bolus that can be swallowed safely, without the danger of stray particles entering the windpipe — but we should not chew for too long because, if we do, excess saliva will weaken the cohesive forces and allow the bolus to fall apart.

Prinz and Lucas predict an optimum number of chews for any particular food. To find this optimum, we have to understand both how the teeth break the food up, and how the saliva makes the fragments stick together. A piece of food in the mouth may or may not be broken into smaller fragments during a particular chewing cycle. The probability depends on its size — smaller fragments are less likely to be broken. If the piece of food does break, it may cleave into two pieces or shatter into many tiny fragments, depending on its toughness (the energy needed to produce unit surface area of crack) and Young's modulus (a measure of stiffness). The same group has already predicted³ that fragmentation depends on the ratio of toughness to Young's modulus, and they confirmed this by experiments with foods ranging from soft cheese to Brazil nuts. Stiff foods of low toughness, such as nuts, are most likely to break into many fragments.

The food is formed into a bolus by tongue movements, which have been shown by X-ray cinematography of people with disks of lead foil glued to their tongues⁴. The cohesion of the bolus is expected to depend on viscous forces — the

forces that make it so difficult to separate two sheets of wet glass. The thinner the layer of liquid between the solid particles, the more strongly they will cohere. Large

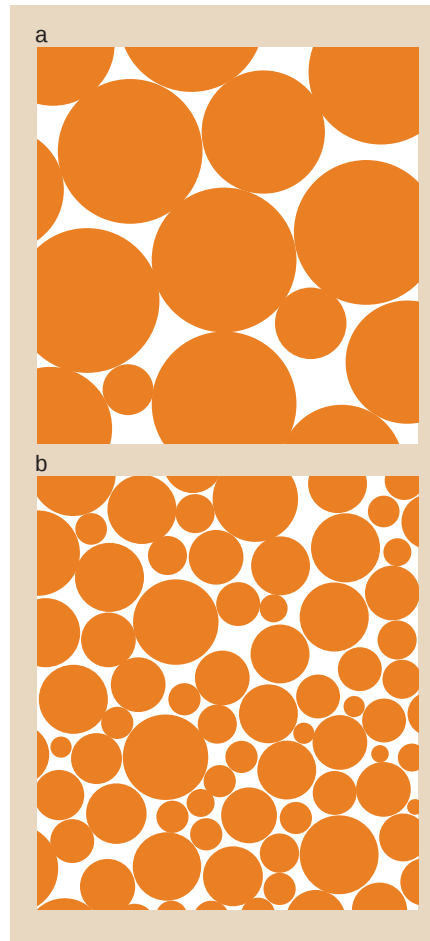


Figure 1 Packing of raw carrot fragments at (a) an early, and (b) a later, stage in a computer simulation of chewing. Prinz and Lucas² studied the distribution of particle sizes after each chew, and they predicted that cohesion of the food fragments would peak after 20–25 chews. In fact, people tested chose to swallow raw carrot after a mean of 31 chews — in reasonable agreement with the predictions. (Adapted from ref. 2.)

particles cannot, in general, be packed close together (Fig. 1a), but smaller particles pack more tightly. It is not necessary for all of the particles to be small, because small particles can fill the spaces between large ones (Fig. 1b). Thus, the bolus is expected to cohere more firmly as chewing proceeds. But later in the chewing process there is too much saliva in the bolus to allow the particles to pack as tightly as they otherwise could, and cohesion falls.

Prinz and Lucas² applied their theory to raw carrot and Brazil nuts. The fragmentation properties of both foods had been measured by previous experiments in which subjects spat the fragments out for analysis after different numbers of chews. The viscosity of saliva was measured, and a typical rate of secretion was assumed. A computer program predicted the distribution of particle sizes after each chew, then generated diagrams like those shown in Fig. 1 to estimate how closely the particles would pack. Although carrots and nuts have very different properties, the program predicted that cohesion would peak after 20–25 chews in each case. Subjects chose to swallow raw carrot after a mean of 31 chews, and Brazil nuts after 25, in reasonable agreement with the theory. When I was a child, my mother used to tell me to chew everything 30 times.

A different optimization criterion has been formulated for herbivorous mammals, whose food intake is limited by the time available for eating⁵. The longer they chew, the more finely the food is ground and the faster it can be digested or fermented in the gut — but prolonged chewing leaves less time for eating. Unlike the work of Prinz and Lucas, this theory takes into account the rates of digestion and fermentation in the gut. It treats the gut as a chain of chemical reactors, and it predicts that optimal chewing times will be longer for foods that can be eaten quickly, that break down only slowly when chewed, and that contain a lot of cellulose. We should not be surprised that cattle chew for about eight hours each day.

For humans, however, food intake is not seriously limited by the time available for eating, and the new model² seems the more relevant. We used to think that we should chew until our food was thoroughly broken up. Now we know that we should chew until it sticks together again. □

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Particle physics

Liberating quarks and gluons

Frank Wilczek

An extraordinary new state of matter, the quark–gluon plasma, may have been produced. In collisions between high-energy heavy nuclei, conditions like those 0.01 seconds after the Big Bang are reproduced — though only on a small scale and briefly. Temperatures of 10^{12} K or more are achieved, roughly ten million times that at the surface of the Sun, or ten thousand times that in the solar core. Theorists predict that under these conditions there is a drastic change in the structure of nuclear matter. The usual description in terms of baryons (such as protons and neutrons) and mesons (such as pions) must be abandoned in favour of a description involving the truly fundamental particles, quarks and gluons. Experiments last year have provided the first substantial evidence that such a change in fact occurs^{1–3}.

To put these developments in perspective, let me sketch some of the background. Quantum chromodynamics (QCD) is the modern theory of the strong interaction⁴ — the most powerful force of nature, responsible for holding atomic nuclei together, and for most of what goes on in high-energy accelerators. QCD is verified by dozens or perhaps hundreds of experimental tests^{5,6}. But it is a most peculiar theory: its fundamental particles, the quarks and gluons, have never been observed in isolation. Indeed, the theory predicts that they never will be.

According to QCD, particles that carry uncompensated colour charge call forth such strong forces that they spontaneously ionize empty space. One might say that they cause a colour lightning storm. When the storm subsides, all the colour charges have been neutralized. The strongly interacting particles that experimenters can actually observe are composites of several quarks and gluons, arranged into structures with compensating colour charges, so that they are neutral overall. The most important types are baryons, which can be constructed from three quarks; antibaryons, constructed from three antiquarks; and mesons, constructed from a quark and an antiquark.

Quarks and gluons themselves carry uncompensated colour charge, and therefore, according to QCD, cannot exist in isolation — they are ‘confined’. Nevertheless, to modern physicists quarks and gluons are quite real and tangible objects, no less than (say) electrons. Indeed, quarks and gluons have quite distinct signatures (Fig. 1). They can do so, despite being confined, because of the special nature of the forces among them. The powerful confinement forces only come into play when colour charges are taken far apart from one another, and they take some time to set in. When the colour balance is disturbed by sudden, small-scale motions, to begin with the colour forces are much weak-

er. This property of QCD is called asymptotic freedom. In a high-energy collision, the key events that control the large-scale flow of energy and momentum are brief, violent accelerations of the particles. Because of asymptotic freedom, these events occur almost as if the quarks and gluons were free and unconfined, so the energy and momentum distribution follows a pattern imprinted by the quarks and gluons (Fig. 1).

In a quark–gluon plasma, liberation of quarks and gluons is taken to a new level. At low particle densities, each coloured particle is bound up with its neutralizing partners, inside some ordinary hadron (Fig. 2a). At high particle densities the hadrons start to overlap, and cease to exist as meaningful individuals. Indeed, a particle needing a mate no longer finds it necessary to stay married to a particular partner, since there are always plenty of eligible singles nearby (Fig. 2b). The meaningful units are then quarks and gluons, not hadrons.

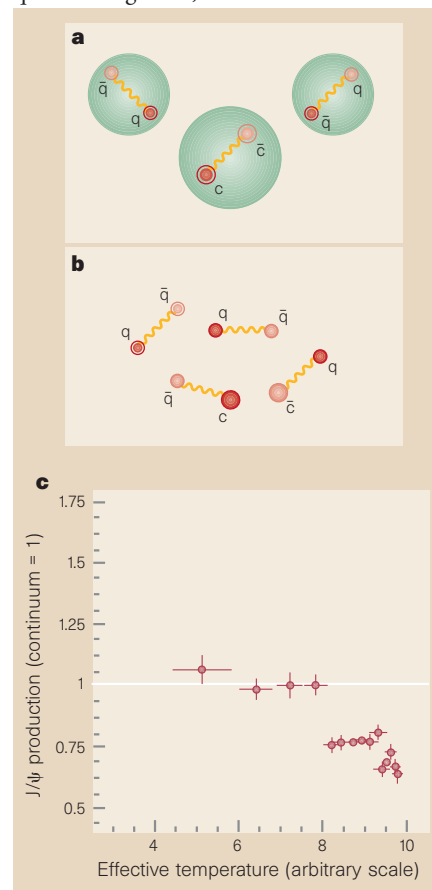


Figure 2 Confinement and deconfinement. When the surrounding quarks and gluons are contained in colour-neutral hadrons (a), the charm quark and antiquark that make up a J/ψ particle must also stay paired, so that their colour charges cancel out. But if the surrounding quarks and gluons run free (b), the components of the J/ψ readily make and break bonds with other particles, and the J/ψ particles dissociate much more quickly. A rapid decrease in J/ψ production with effective temperature has been seen by the NA50 experiment¹⁰ at CERN (c).

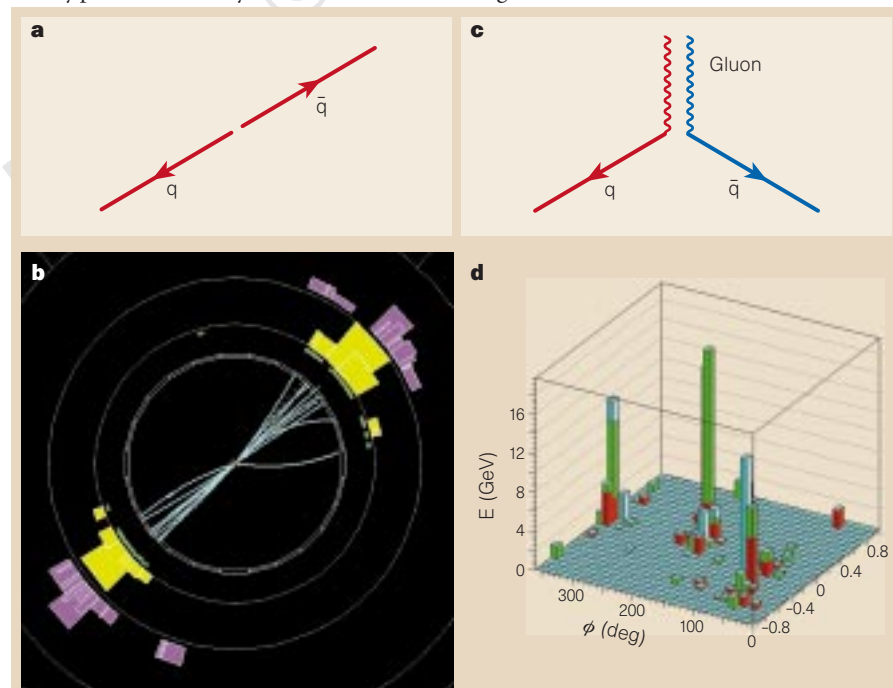


Figure 1 Realization of quarks and gluons as jets. A Z-boson decay into quark and antiquark (a) is reflected in the jets of compound particles observed (b). When the Z-boson instead decays into quark, antiquark and gluon (c), the observed energy and angle distribution of the products (d) again follows the pattern set by the underlying quarks and gluons. (Parts b and d from ref. 9.)

Theorists calculate that this drastic change in the structure of matter sets in over a narrow range of temperatures centred around 10^{12} K. To gauge the change, it is instructive to compare the degrees of freedom—the number of different particles that energy can go to. On the hadronic side of the transition, the important particles at these temperatures are just the pions (the other hadrons being too heavy). These are spinless particles and come in three types—with positive, negative or zero electric charge. On the quark–gluon side, there are three colours of quarks of three different types (up, down and strange—the other quarks are too massive to play a part) and each has two possible spin directions. Taking into account antiquarks, we find $3 \times 3 \times 2 \times 2 = 36$ quark degrees of freedom. In addition, there are eight gluons each with two possible spin directions—thus 54 degrees of freedom altogether, compared with the previous three. A direct consequence is that a given input of energy will raise the temperature of a quark–gluon plasma much less than it would the hadronic gas, as the energy has to be shared by many more particles.

Despite the dramatic nature of these predicted changes, it is not easy to establish experimentally that one has produced a quark–gluon plasma. Difficulties arise because the number of particles reaching the detectors after a heavy ion collision is extremely large, and because the plasma, even if produced, has only a fleeting existence in a very small region.

The experimenters are like inspectors who must examine the residue of a great explosion to determine if it was due to conventional or nuclear weapons (or perhaps a meteorite). Ambitious responses to this challenge are being mounted at CERN and at Brookhaven, where the heavy-ion accelerator RHIC will come into operation next summer.

Already, CERN has seen what might be the first harbinger of the quark–gluon plasma. Charm–quark/charm–antiquark pairs, making up the J/ψ family of particles, seem to find it much more difficult to stay paired once the energy in a fireball exceeds a threshold value (Fig. 2c). This certainly suggests the deconfinement mentioned above. It has been advocated for some time as a signature of the quark–gluon plasma. Even though the issue is muddled by the fact that the J/ψ particles will be buffeted more at higher temperature whether one has hadrons or quark–gluon plasma, nevertheless the apparent sharpness of the threshold, and other details, point towards the plasma. What makes the latest results³ especially intriguing is that they are the first that sceptical theorists⁷ have not been able to explain without invoking a quark–gluon plasma.

A big question left open by these experiments is whether the transition from normal matter to quark–gluon plasma, as a function

of temperature, is continuous or truly abrupt. If it is abrupt (in the language of phase transitions, first order), superheating and supercooling are possible, and could trigger explosive instabilities. If such events occurred in the early Universe, they must have appreciably perturbed its evolution.

We anticipate other relics of the quark–gluon plasma created in accelerators. An especially intriguing possibility is that the quark–antiquark condensate which normally fills space could reassemble incorrectly, forming a domain analogous to domains in magnets. When such a domain snaps back into place, it will release a laser-like pion beam⁸.

More prosaically, it would be reassuring to see the predicted high specific heat, and the associated increase in multiplicity of particles. In particular, strange quarks and antiquarks

are much lighter and therefore much easier to produce than the K mesons in which they are normally confined. So events following the creation of a quark–gluon plasma should be especially strange, in more ways than one. □

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Neurobiology

Phantoms of the brain

Jon H. Kaas

The brain often reorganizes itself after damage to some of its sensory inputs, so that neurons that were responsive to the missing inputs come to respond to remaining inputs¹. After the loss of somatosensory input from the hand, for example, the region of the somatosensory cortex on the opposite side of the brain that is normally responsive to touch on the hand becomes responsive, over months of recovery, to touch on the face or arm^{2–6}.

When the brain reorganizes in this way, do the newly reactivated neurons signal that the sensations are coming from the location of the stimulated skin, or do they signal instead the location of their original but missing source of activation? This question has been tackled by Karen Davis and colleagues (page 385 of this issue⁷) by recording and stimulating brain responses with microelectrodes placed in the somatosensory thalamus of patients with missing limbs (Fig. 1, overleaf).

People with amputations often have the feeling that the missing limb is still present as a so-called phantom limb⁸. Furthermore, sensations on the missing limb can sometimes be evoked by touching 'trigger zones' on other parts of the body. For example, touching the face or remaining upper arm on the side of an arm amputee may produce sensations both of those body parts and of the missing hand^{9,10}. A logical interpretation of these trigger zones is that touching the arm or the face activates neurons in the arm or the face territories in the brain, and the territories normally devoted to the hand.

According to this view, this type of brain reorganization is not beneficial, but instead contributes to the misperception that something is touching the phantom hand. Another possibility, however, is that the reactivated

neurons devoted to a missing hand or foot become recalibrated by experience so that they come to signal stimuli on remaining body parts such as the hand or face. This, of course, would not explain trigger zones, but it would mean that the extensive brain reorganization that follows amputation is potentially useful.

People without amputations report appropriately localized sensations when sensory representations in the brain are stimulated electrically¹¹. As part of a therapeutic procedure for amputees with pain, Davis and co-workers⁷ placed microelectrodes in normal parts of the somatosensory thalamus and in that part of the thalamus where neurons previously would have been activated by stimulating the missing limb. The investigators determined the regions of skin where light touch activated neurons recorded at various electrode locations, thereby defining the receptive fields of those neurons; and they electrically stimulated the same or nearby neurons to produce sensations, thus defining sensation fields.

In the normal, undeprived portions of the somatosensory thalamus, neurons had matching receptive fields and sensation fields. But in some amputees, those with notable phantom sensations, stimulating neurons with receptive fields on the stump of the missing limb produced sensations referred to the missing limb (Fig. 1). Thus, the brain had reorganized so that the territory of the missing limb in the thalamus had become responsive to the sensory inputs from the stump of the arm, whereas the activation of neurons in this territory continued to signal sensations on the missing limb.

This does not tell us how or where the sensations are generated, because the activated

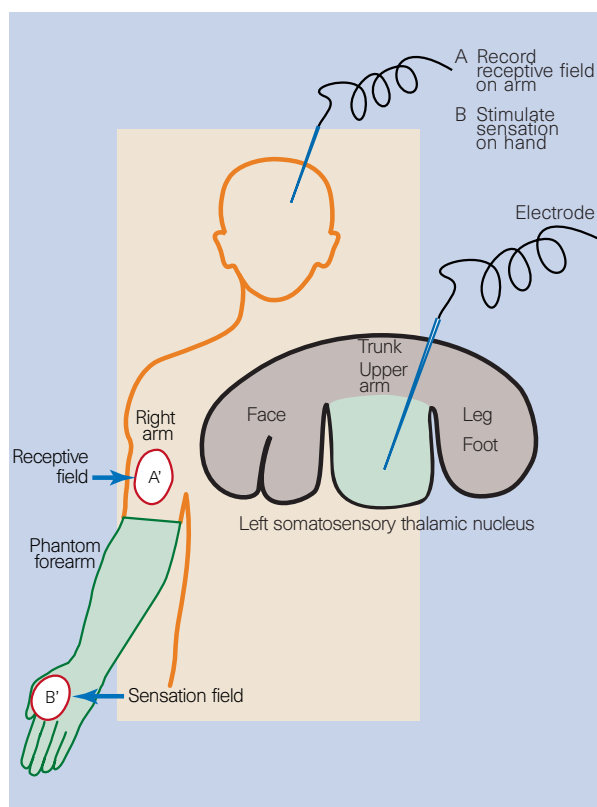


Figure 1 Evidence of brain reorganization without respecification. The studies of Davis *et al.*⁷, discussed here, involved recording and electrically stimulating neurons in the somatosensory thalamus of patients with a missing forearm or lower leg (not shown). When the electrode is inserted into the portion of the somatosensory thalamus where neurons are normally activated by touch on the hand, recordings (A) reveal receptive fields on the stump of the amputated limb (A' on the right arm). Thus, the thalamus has reorganized so that the hand region responds to the upper arm. When the same neurons are electrically stimulated (B), sensations are felt on the missing hand (sensation field, B'). So the reorganized hand portion of the thalamus continues to signal the hand's existence.

neurons in the thalamus in turn activate neurons elsewhere in the brain. But we now know that, for at least some people, deprived but reactivated neurons do not take on new and appropriate functions. Instead, these neurons continue to carry out their original roles. However, mismatched receptive and sensation fields were not found in all patients, suggesting that sometimes the reactivated neurons recalibrated to signal stump locations rather than locations on the missing limb.

The results of Davis and co-workers are consistent with limited observations of Woolsey *et al.*¹² on the effects of stimulating somatosensory cortex in a patient with phantom leg pain. Electrical stimulation of the leg area of somatosensory cortex produced the sensations in the phantom leg. No studies were carried out on the possible reorganization of this cortex, but the observation that sensations were referred to the phantom leg indicates that that part of the cortex continued to signal the existence of the missing leg. We can conclude from these studies that neurons in the brain can retain their original functions long after they have had time to adopt new ones.

The thalamic stimulations and recordings in amputees provide support for another important conclusion. Most of the evidence for brain reorganization after injury or altered experience has come from studies of the more accessible sensory representations of the cortex on the surface of the brain. Information from receptors in the skin is relayed through sensory nerves to the lower brain stem, then to the thalamus, and next to the cortex. Depending on the circumstances

of injury or experience, reorganizations of cortical maps could depend on modifications of neural circuitry that occur in the cortex, subcortical stations, or both. In monkeys with long-standing therapeutic forelimb amputation, the hand region of cortex is activated by intact inputs from the upper arm and face³. In these same monkeys, nerve fibres from the upper arm appear to have sprouted in the lower brain stem to innervate neurons normally contacted by nerve fibres from the missing hand.

From this it seems that the growth of new nerve terminals at the level of the first relay station activates the deprived brain-stem neurons, which project to and activate deprived neurons of the somatosensory thalamus, which in turn relay to the cortex. The extensive reactivation of deprived portions of the human thalamus demonstrates that there is a subcortical locus for much of the reorganization that follows limb amputation. □

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100 YEARS AGO

"Rev. C. L. Dodgson" – A formidable champion of Euclidean methods in the elementary teaching of geometry has just passed away after a short illness. ... Without stint of labour he submitted to rigid logical analysis every text-book on the subject that came to his notice, undismayed by their surprising number, the result being the amusing and, at the same time, deep "Euclid and his Modern Rivals," published in 1879, in which he demonstrated the logical superiority of Euclid's method over all the others examined. ... He invented a new method of evaluating determinants, which is published in the *Proceedings of the Royal Society* for 1866, and also a method (which was published in *NATURE*) of easily determining the day of the week corresponding to any date. In October last he described in *NATURE* a brief method of dividing a given number by 9 or 11; and a second paper on the same subject, which appears in our correspondence columns this week, probably represents his latest contribution to mathematics. ... Mr. Dodgson's mind was essentially logical, in spite of the whimsical humour which has endeared "Lewis Carroll" to every boy and girl – nay, every adult – in the kingdom. A shy and retiring man, he was to his friends a most charming companion, overflowing with the quaintest of humour, and one whose love for children was typical of himself, and whom to know was to love.
From *Nature* 20 January 1898.

50 YEARS AGO

The Royal Society Empire Scientific Conference held in June 1946 considered and approved a resolution advocating that where scientific papers or text-books are expressed primarily in British units, provision should be made for the inclusion of metric equivalents or conversion factors. ... Sir Charles Darwin in opening the meeting emphasized that the matter to be discussed was one of intelligibility only, and had nothing to do with the introduction of the metric system in Britain. By making it possible for the foreigner to convert British units immediately into metric, publishers of scientific papers and books would assist Britain to attain the position of the centre of science in Europe.
From *Nature* 24 January 1948.

Crystallography

Some are less equal than others

Alan Mackay

Why is it dark at night? This is Olber's Paradox. The modern explanation for it is that the Universe is expanding; but in 1922 C. V. L. Charlier suggested another, ingenious solution: the Universe might be infinite and hierarchical, with galaxies of galaxies of galaxies. There would be no privileged centre and all viewpoints would be quasi-equivalent, yet the density of this Universe would tend to zero with increasing scale. A similar idea might apply to the microcosmos of atomic arrangements: clusters of clusters are an alternative to strict crystalline arrangements, and could form a new type of condensed matter. On page 376 of this issue¹, Hubert *et al.* report a structure with this theme — clustered icosahedral particles of boron suboxide.

The mathematical simplicity of infinite crystal lattices has beguiled crystallographers, with its absolute identity of lattice points. The classification system of crystals is based on the 230 symmetries allowed by that assumption — that an infinite number of asymmetric units are to be arranged so that

their surroundings are absolutely identical. The assumption of crystallinity is necessary for the recovery of phase information in single-crystal X-ray diffraction, which is the principal tool of crystallography, so sharp diffraction patterns can be interpreted in terms of infinite crystals. But hierarchy could offer an alternative to lattice repetition, in providing an assembly of atoms with an infinite number of almost identical, or quasi-equivalent, sites.

This principle of quasi-equivalence was formulated by Caspar and Klug² when they considered how polio virus (icosahedral particles of 60 subunits) could form crystals of cubic symmetry³ — as indicated by their diffraction pattern. The solution found by nature for polio is surprisingly like Linus Pauling's structure of $Mg_{32}(Al,Zn)_{49}$: the units are locally icosahedral clusters arranged on a translation lattice.

Perhaps this should not be surprising: identical units do not have to have identical surroundings, and natural materials are physical and not mathematical objects. Bio-

logical structures such as collagen are predominantly hierarchical in the same way as rope: fibres are twisted into strands, which are then twisted into bigger strands, and so on for several levels.

Hierarchy has now also appeared as a building principle in a class of inorganic materials, the quasicrystals. These are solids with fivefold symmetry (as betrayed by their diffraction patterns) — a symmetry impossible for a conventional crystal.

F. C. Frank noted that the densest packing of 12 spheres around a central sphere is icosahedral, and J. D. Bernal, following Charlier, asked whether this rule could be continued hierarchically. The problem is to find further rules to fill in the gaps so as to keep the overall density finite. Model-building experiments produced an icosahedral shell packing⁴ which, for the first few layers, was close to a packing of 13 icosahedral clusters, each of 13 spheres — and indeed β -rhombohedral boron was found in 1970 to contain such $B_{12}(B_{12})_{12}$ units.

Another way to make icosahedra is found in multiply-twinned particles of silver and gold, where 20 face-centred-cubic regions are joined, with slight distortion, into icosahedra. These have been observed⁵ by electron microscopy from about 1964, and many other icosahedral clusters have since been found. Their size is limited by the increasing strain as successive layers are added, the spacing of spheres in the layers being some 5% greater than in close packing, so a transition from icosahedron to cuboctahedron will probably occur at a certain size.

The first theory of hierarchical packing came from the tiling of pentagons in two dimensions. Consideration of how the gaps were to be filled in led to Penrose tiling⁶ in two and three dimensions — a mathematical pattern that has the geometrical properties required of a quasicrystal. In three dimensions, Penrose tiles are obtuse and acute rhombohedra with angles of 116.6° and 63.4° .

Significantly, the 20 tetrahedra making up the multiple twin in gold and silver must have inter-edge angles at the centre of the cluster of 63.43° (instead of 60° for regular tetrahedra). And now Hubert *et al.*¹ have found that boron suboxide crystals (B_6O) are rhombohedral, with an inter-edge angle of 63.1° , and that these crystals occur as icosahedral twins. Twenty rhombohedral unit cells fit together snugly at a common vertex without dislocations at the interfaces, and the structure can continue outwards indefinitely, particles of some 10^{12} atoms being observed (Fig. 1).

Without dislocated grain boundaries, glide planes in this boron suboxide are locked and so the particles are very hard, promising technical applications. And these aggregates are far larger (up to $20\ \mu\text{m}$) than those observed hitherto for multiply-twinned particles. B_6O and various other

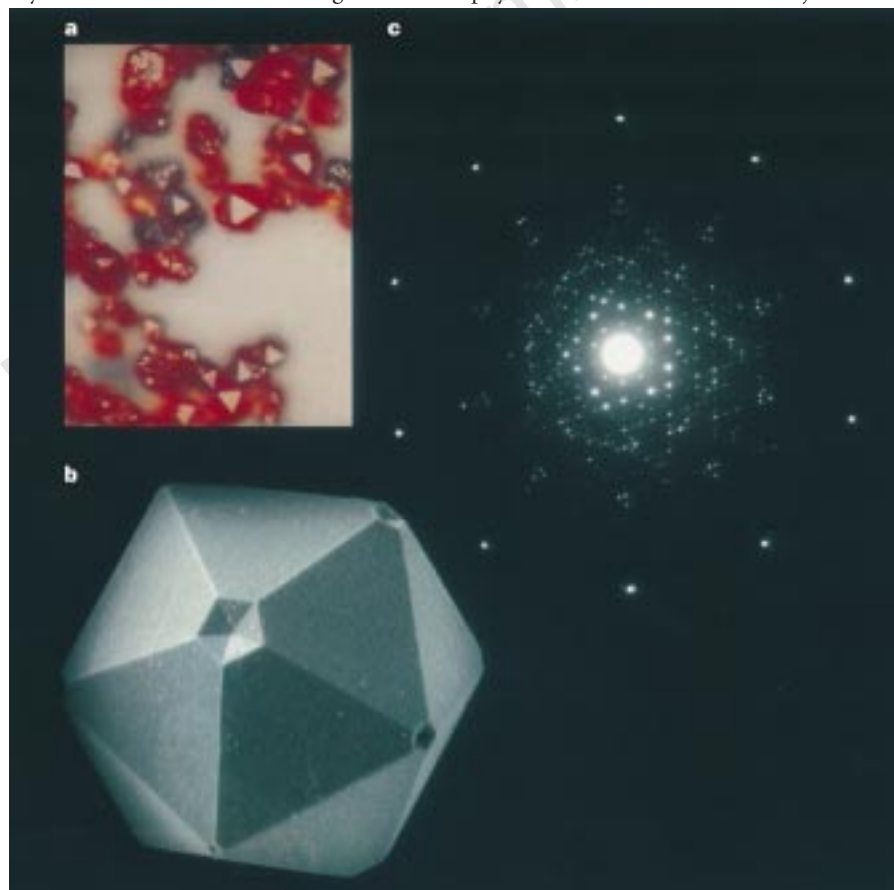


Figure 1 Clusters of boron suboxide, B_6O (a, photograph; b, electron micrograph) and its X-ray diffraction pattern (c). These icosahedral shapes, up to about $15\ \mu\text{m}$ across in the photograph, are not crystals, but hierarchical clusters with a fivefold symmetry forbidden to crystals. B_6O may also be a useful engineering material, because of its hardness.

boron compounds show marked tendencies towards icosahedral packing and it was thought, before quasicrystals of Al_6Mn were reported, that boron would be the best candidate for forming a three-dimensional Penrose tiling, as the unit cell of B_6O is the Penrose acute rhombohedron.

These boron suboxide particles are not quasicrystals, but they are an important step away from the 230 space groups towards a more general type of structure.

True quasicrystals can probably also be described as icosahedral clusters, themselves clustered icosahedrally in hierarchical levels, the gaps being filled by the overlapping of these clusters. Quasicrystals are a further step away from conventional crystals, because they have many centres of local icosahedral symmetry, whereas a boron suboxide particle has only one.

As more varied structures appear —

especially to the electron microscope, which does not depend on the assumption that many copies of the same unit can form only crystals — we can escape from the preconceptions engendered by the immense success of X-ray single-crystal structure analysis. We must expect many still more varied structures to lie outside the austere dominion of classical crystallography. □

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Genetic recombination

From competition to collaboration

Roland Kanaar and Jan H. J. Hoeijmakers

Politics and science furnish many examples of the dramatically different effects of competition and collaboration. Similar phenomena occur at the molecular level in nature, and four reports^{1–4} (three of them in this issue^{2–4}, beginning on page 401) demonstrate the point. They show that competition between two proteins required for genetic recombination is turned into fruitful collaboration by a third participant, the Rad52 protein.

Genetic recombination, the exchange of information between DNA chains, accomplishes two seemingly conflicting tasks — generation of genetic diversity within species, and maintenance of genetic stability by repairing DNA damage. Whether recombination results in diversity or stability depends on whether the exchanges occur between homologous chromosomes during meiosis or between identical sister chromatids. Clinically, genetic recombination has attracted attention because of possible cross-talk between the breast-cancer-susceptibility genes, *BRCA1* and *BRCA2*, and the recombination machinery⁵; biologically, its importance is underscored by the conservation of its salient features from fungi to humans.

At the core of recombination is the search for homologous DNA followed by exchange of DNA strands (Fig. 1). A common initiator is a DNA double-strand break that is processed to expose regions of single-stranded DNA (ssDNA). In eukaryotes, the Rad51 protein coats the ssDNA to form a filament that scans the genome for a homologous double-stranded DNA (dsDNA) sequence (in a mammalian nucleus, which contains

about 6×10^9 base pairs, this must be an especially arduous task). On completing this quest, the ssDNA-containing filament and the intact dsDNA form a joint molecule before strand exchange can occur.

But how, *in vivo*, does the filament assemble despite being subject to many competing reactions? An ssDNA-binding protein known as RPA (replication protein A) is required, presumably to remove secondary structure from the ssDNA to allow for efficient filament formation by Rad51, but it also competes with Rad51 for ssDNA binding. And although dsDNA is a substrate for

the reaction, it binds Rad51, thereby inhibiting filament formation. From genetic studies it was clear that another protein, Rad52, was a major player in these events, but unlike Rad51 and RPA its molecular function remained elusive. The new, biochemical, studies reveal the effect of Rad52.

Three of the reports^{1–3} deal with the budding yeast *Saccharomyces cerevisiae*. They show that although RPA is required for Rad51-promoted strand exchange, it inhibits exchange when incubated simultaneously with Rad51 and ssDNA. Inhibition is overcome when Rad52 is incubated together with Rad51, RPA and ssDNA, followed by the addition of homologous dsDNA.

Rad52 is ideally suited for this job as mediator between Rad51 and RPA, because it interacts with both proteins^{1,6} and binds ssDNA^{4,7}. The mechanism is not yet clear, but Rad52 could function in several, not mutually exclusive, ways. First, it could increase the cooperativity of Rad51 binding to ssDNA. Second, it could enhance the dissociation of RPA from ssDNA and promote RPA transfer to the displaced strand, preventing reversion of strand exchange. Third, the ability of Rad52 to increase the annealing rate of complementary ssDNAs (ref. 7) could help Rad51 initiate joint molecule formation.

The other report⁴ in this issue concerns human Rad52. The authors show that joint molecule formation does not occur when human Rad51 is present at subsaturating amounts. But when ssDNA is preincubated with human Rad52, followed by the sequential addition of subsaturating amounts of human Rad51 and dsDNA, joint molecule formation proceeds efficiently. These observations support the idea that human Rad52 increases the cooperativity of human Rad51

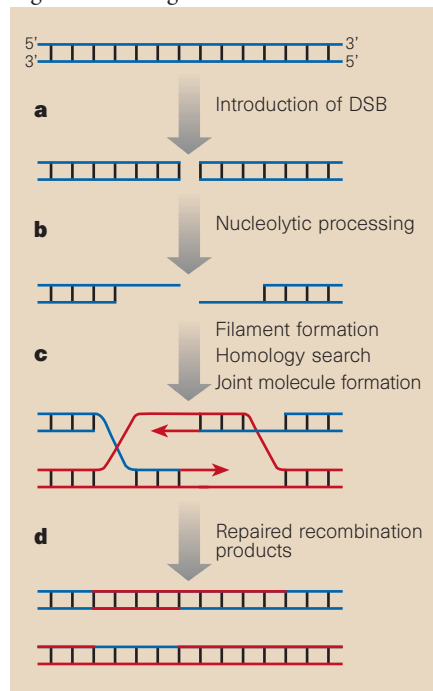


Figure 1 The function of Rad52 in genetic recombination. a, Recombination can be initiated by a double-strand break (DSB) that may be caused by an endonuclease or a DNA-damaging agent. b, The DNA is processed at the site of the break to yield regions of single-stranded DNA. c, Rad51, assisted by replication protein A (RPA), coats the single-stranded DNA to form a filament that searches for homologous sequences (on the homologous chromosome or the sister chromatid) and, when it finds them, initiates the formation of a joint molecule. Four studies^{1–4} now show that Rad52 stimulates homologous pairing by Rad51. d, The break is repaired by DNA synthesis (arrows in c) using the intact strands as templates. Following branch migration and resolution, repaired recombination products are released.

Table 1 DNA strand exchange: the difference in the details

Recombination protein	Bacteriophage T4	<i>E. coli</i>	<i>S. cerevisiae</i> & <i>H. sapiens</i>	Homologues in <i>S. cerevisiae</i>
Strand exchange	UvsX	RecA	Rad51	Rad55, Rad57, DMC1
Single-strand DNA binding	Gene 32 protein	SSB	RPA	None
Mediator	UvsY	Not required	Rad52	Rad59

binding to ssDNA, thereby converting discontinuous stretches of Rad51 on ssDNA into one functional contiguous filament.

These new results reveal an interesting parallel between eukaryotic and prokaryotic genetic recombination. Bacteriophage T4 also uses a mediator, the UvsY protein, to stimulate strand exchange by UvsX and the T4 ssDNA-binding protein (see Table 1)⁸. The requirement for a mediator, however, is not universal because the *Escherichia coli* RecA protein, the bacterial homologue of Rad51, does not need one. So even though strand-exchange proteins are structurally and functionally highly conserved, the details of their actions differ (see Table 1). Yeast and human Rad51, and T4 UvsX, have no clear preference for binding to ssDNA or dsDNA, whereas *E. coli* RecA strongly prefers ssDNA. This property can eliminate competition on two fronts — RecA can itself displace the *E. coli* ssDNA-binding protein without the help of a mediator, and can overcome the competition by dsDNA for filament formation.

The ramifications of the four papers^{1–4} go further, for *S. cerevisiae* contains a second RAD52 homologue, RAD59, which is required for Rad51-independent recombination between intra-chromosomal inverted repeats⁹. If RAD52 homologues also exist in other species, it would help explain the dramatically different effects of RAD52 mutations in *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* and mouse cells. Although the efficiency of recombination is reduced by more than three orders of magnitude in the *S. cerevisiae* RAD52 mutant, it is only twofold lower in the corresponding *S. pombe* mutant¹⁰, and only slightly affected in mouse RAD52 knockout embryonic stem cells (T. Rijkers and A. Pastink, personal communication).

The explanation could be that some functions of *S. cerevisiae* Rad52 can also be assumed by Rad59 in *S. pombe* and mammals. Another possibility is that different Rad52 homologues act as mediators for different Rad51 homologues, for eukaryotes express multiple versions of RAD51. In *S.*

cerevisiae, these variations on the Rad51 theme appear to assist Rad51-promoted strand exchange or to be involved in meiosis-specific processes (see Table 1). In mammals their functions are less clear. But, among other things¹¹, they could be important at the filament ends, which might have different properties to those of the filament body; in this respect, their role would be somewhat analogous to that of the *E. coli* RecF and RecR proteins in post-replication repair¹².

The new reports^{1–4} show that mediation by Rad52 is required during the critical, early steps in genetic recombination. But another question remains — because Rad51 binds with similar efficiencies to ssDNA and dsDNA, how is the inhibitory effect of dsDNA on filament formation overcome? The answer might lie in the requirement for additional protein factors. A candidate is Rad54, whose function is still unknown, but it interacts with Rad51 and genetically it is in the same epistasis group as Rad51 and Rad52. Moreover, there must be much more to come in the Rad52 story because it also features in Rad51-independent recombination. □

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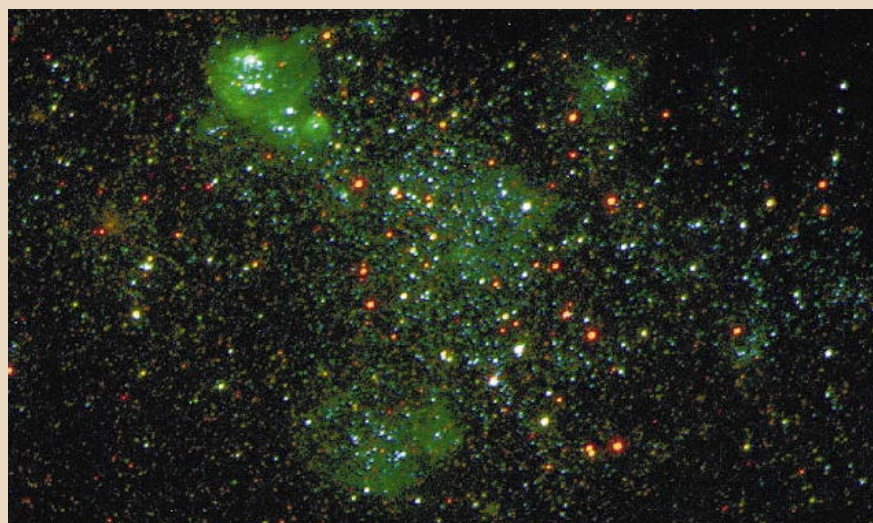
Galaxy evolution

Red giants in blue dwarfs

Are all galaxies old? Most that we observe certainly are, as they contain some stars almost as old as the Universe. But blue compact dwarf galaxies (BCDs) had been thought younger — rare baby galaxies in among the adults. Now that is called into doubt by one BCD that contains old red giant stars.

The first generation of stars in a galaxy forms from primordial gas produced in the Big Bang, mostly hydrogen and helium. They burn this by nuclear fusion, and some explode, sending out heavier elements that end up in younger stars such as the Sun. But the BCDs contain few heavy elements (as determined from their spectra), and so there is little evidence for an early generation of stars in these galaxies — they appear, in this respect, to be truly new galaxies.

On the right is a small galaxy called VII Zw 403, about fifteen million light years away — close in cosmological terms, but until now too far away for telescopes to resolve the tell-tale red giants. So R. E. Schulte-Ladbeck, M. M. Crone and U. Hopp delved into the data archives of the Hubble Space Telescope (*Astrophys. J.*



493, L23–L26; 1998). The blue stars and the bright red supergiants are young; but the fainter, ordinary red giants, dotted about over the whole image, are old, in a late stage of stellar evolution that our Sun will also undergo.

There is no doubt that BCDs are undergoing rapid star formation now; and their lack of heavy elements suggests that

star formation may have paused between formation of the earliest and the new generation of stars.

So one BCD has admitted its age. Will the others follow suit? Schulte-Ladbeck and Crone have now won time with Hubble to look closely at four more galaxies, so we may soon know.

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Palaeoclimatology

A glimpse of the glacial

Thomas F. Stocker

During most of the past 100,000 years, temperatures on Earth were much colder than they are now and climate was very unstable. About 21,000 years ago that climatic period culminated in the Last Glacial Maximum (LGM), when about 50 million km³ of water was locked in huge ice sheets, lowering sea level by more than 120 metres. Climate during the LGM was clearly very different from what it is today. But how different? This is the subject of the paper by Ganopolski *et al.* on page 351 of this issue¹.

Using a properly tuned, simplified, coupled ocean–atmosphere climate model, they first verify the model's ability to simulate modern climate on the global scale. After adapting insolation, concentrations of atmospheric greenhouse gases and ice-sheet distribution to values typical for the LGM, they find that the same model yields a stable climatic state with other atmospheric and oceanic characteristics that are reminiscent of the LGM. The simulated changes are broadly consistent with what we know from decades of invaluable analyses of marine sediments, polar and tropical ice cores, tree rings and groundwater — all of which are archives that record past climatic changes.

Why should we be interested in simulating the climate of the LGM, when that state is unlikely to return for another 50,000 years² and Earth's climate will instead most probably warm considerably? There are four rea-

sons. First, climate models must be able to simulate the full range of dynamical behaviour of the climate system, and so the LGM or transient and extreme climatic periods are the most critical tests they can be exposed to. Second, models that estimate future changes must be consistent with the sensitivity of the climate system to altered forcing parameters. Third, these models, if correct, can provide a more detailed picture of past changes for regions or parameters for which no suitable palaeoclimate archives are available. Finally, this will eventually contribute to a quantitative, model-based interpretation of palaeoclimatic proxy data.

Ganopolski *et al.* abandon the classical approach of using comprehensive three-dimensional general circulation models of one of the two main components of the climate system (the atmosphere or the ocean), while using a simplified representation of the other. Instead, they build on the progress made in developing zonally averaged climate models^{3,4}, which have become important tools in palaeoclimatic research, and combine it with a new atmospheric model component. Such models permit the numbers of runs required for optimal tuning. This means that loosely constrained model parameters or incompletely known boundary conditions can be varied in such a way that the relevant atmosphere–ocean exchange fluxes of momentum, heat and fresh water,

simulated by the respective model components, are brought into adequate agreement.

Because of limitations on computer time, such systematic fine-tuning is not yet possible for coupled three-dimensional models. So most of these models drift to unrealistic states, and flux corrections must be applied. This remedy is undesirable but permissible for small and linear excursions from a well-defined climate state. But caution is called for when modelling climates as different from today's as the LGM.

The crucial component for a successful simulation is the hydrological cycle, which is notoriously difficult to simulate. Water vapour is the most important greenhouse gas, and its reduction in level contributes significantly to the global cooling of 6.2 °C in Ganopolski and colleagues' model. This is colder than the usually accepted estimates of about 4 °C, but it is consistent with recent reconstructions of significantly lower temperatures for the LGM at high and low latitudes^{5–7}.

The hydrological cycle also influences the circulation of the deep ocean. Changes in patterns of evaporation and precipitation determine the location of deep-water formation and the mix of waters from northern and southern origin in the North Atlantic⁸. Reduction in high-latitude precipitation, and an even bigger decrease in evaporation due to a larger extent of the ice cover in the northern North Atlantic (the ice margin moves from 75° N to 55° N in the model), mean that a surface freshwater anomaly can develop; in consequence, the area of deep-water formation in the North Atlantic also moves about 20° south. The density of the sinking waters is reduced, with the consequence that they penetrate less deeply. In this way, water masses of southern origin can extend far north in the Atlantic basin and fill much of the deep Atlantic. This meridional shift in the distribution of water mass below 1,000 m, seen in the model, is consistent with palaeoceanographic evidence from stable carbon isotope measurements on benthic foraminifera⁹.

As well as strengthening our confidence in reconstruction of proxy data, coupled climate models are also crucial in assessing hypotheses on which less complete models are based. Ganopolski *et al.* find that, during the LGM, the oceanic meridional heat flux in the Atlantic was very different from today. Although this is not surprising (sea-surface temperatures were lower¹⁰, and sites of deep-water formation and sea-ice margins moved south), it contradicts recent assumptions used for atmosphere-only models¹¹. The reduction of heat delivery into the North Atlantic by northward-flowing waters is also expected, given the extremely cold temperatures on Greenland during the LGM (ref. 5). This leads to a pronounced asymmetry in cooling for the LGM, with a fall of more than

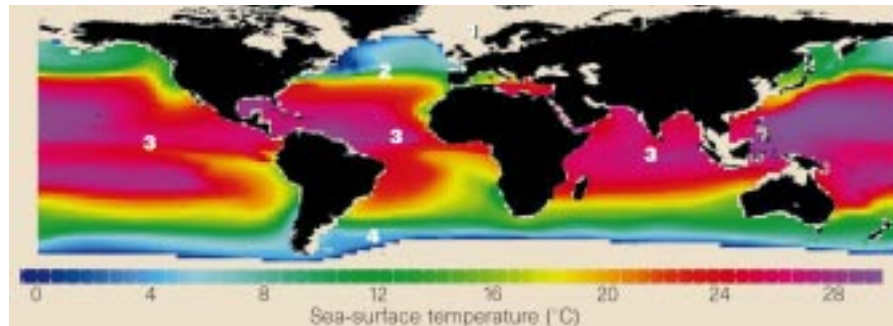


Figure 1 CLIMAP¹⁰ reconstruction of sea-surface temperature in August at the Last Glacial Maximum (LGM), 21,000 years ago. Four areas that pose crucial questions for climate reconstruction by numerical models are indicated as follows. 1, Are the Nordic Seas ice-free during the summer¹²? 2, What are the sea-surface salinities in the area of LGM deep-water formation^{16,17}? 3, What is the reduction of sea-surface temperature in the tropical ocean^{7,18}? 4, What are the surface-water characteristics in the Southern Ocean during the LGM? Other questions concern how the atmospheric temperature gradient changes with time, continental temperatures, the distribution of tracers and isotopes in the ocean, the water-mass mix, and the location of areas of deep-water formation and their seasonality. (Reproduced from ref. 19.)

20 °C in northern polar regions but only 5 °C in the south.

Much remains for palaeoclimatologists and palaeomodellers to do. We need longer data sets with higher time resolution; careful (re-)calibration of proxy indicators; better dating of records, and improved synchronization of records that are located far apart; new analytical techniques; and, not least, coupled low-order and three-dimensional climate models with well-tested parametrizations¹².

Figure 1 outlines four areas of the globe where important issues have to be resolved. The time is clearly ripe for another look at CLIMAP¹⁰, the last large-scale integrated project to tackle the LGM, one which incorporates the vast amount of palaeoclimatological data accumulated over the past 20 years. Moreover, the ocean interior requires continued attention, and initiatives such as IMAGES (International Marine Global Change Study)¹³, or the more encompassing PAGES (Past Global Changes)¹⁴, are invaluable in this respect. After all, research into Earth's climatic history is a cornerstone of building the better computer models that are so urgently needed to assess what the future may hold. □

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Alzheimer's disease

A technical KO of amyloid- β peptide

Christian Haass and Dennis J. Selkoe

Research into the molecular mechanisms of Alzheimer's disease — the most common late-life dementia — continues to illuminate intriguing issues in protein biology, while seeking to identify therapeutic targets. These twin outcomes are exemplified by the exciting results of De Strooper and co-workers¹, reported on page 387 of this issue. The authors show that the presenilin-1 (PS1) gene, mutations in which cause an aggressive form of early-onset familial Alzheimer's disease^{2,3}, regulates the unusual intramembranous proteolysis of the amyloid precursor protein (APP). PS1 might, therefore, serve as an unexpected target for drugs against the disease.

In families with autosomal dominant Alzheimer's disease, mutations in three different genes — APP, PS1 and PS2 — have been identified^{2,3}. Missense mutations in the APP gene are located at, or close to, the recognition sites of the APP-cleaving secretases (α , β and γ ; Fig. 1). These mutations lead to increased production of amyloid- β peptide, particularly its amyloidogenic 42-residue form, the amyloid- β (1–42) peptide, which accumulates in the amyloid plaques of both 'sporadic' and familial cases of Alzheimer's disease³. Whereas mutations in APP are extremely rare, numerous mutations have been observed in the PS1 and PS2 genes^{2,3} (which are homologous to each other). All of the presenilin mutations analysed so far increase the levels of

secreted amyloid- β (1–42) peptide, even presymptomatically, supporting a central role for amyloid- β peptide in the pathogenesis of Alzheimer's disease^{2,3}.

Presenilin proteins span the membrane six to eight times. They are proteolytically processed to produce stable amino- and carboxy-terminal fragments², which form a heterodimeric complex that seems to be biologically relevant (Fig. 1)⁴. Work on the nematode *Caenorhabditis elegans* indicates that presenilins are involved physiologically in cell-fate decisions through the Notch signalling pathway². In support of this, deletion of the PS1 gene in mice produces a fatal developmental defect that closely resembles the phenotype seen in *Notch* knockout mice^{5,6}.

De Strooper *et al.*¹ studied mice lacking both PS1 alleles (PS1^{−/−}). Previous work by this group⁷ showed that, in mouse cells, endogenous APP is poorly processed into amyloid- β peptide, whereas the human APP sequence is efficiently cleaved. To allow generation of amyloid- β peptide in the PS1^{−/−} background to be analysed quantitatively, the authors infected hippocampal neurons cultured from the knockout embryos with a recombinant Semliki Forest virus encoding human APP. Strikingly, these cells produced substantially (~80%) less amyloid- β peptide, and the related peptide p3, than did the same construct infected into cells expressing

PS1. (The residual amyloid- β peptide/p3 may have resulted from continued expression of the homologous PS2 gene.)

In contrast to the presenilin mutations that are responsible for familial Alzheimer's disease — and specifically increase generation of amyloid- β (1–42) peptide — the lack of PS1 reduced production of the 40- and 42-residue forms of amyloid- β peptide equally (Fig. 1). Carboxy-terminal fragments of APP beginning at the β - and α -secretase sites accumulated to high levels in lysates of the PS1^{−/−} neurons. But, in neurons from normal mice, these fragments were efficiently cleaved by γ -secretase, giving rise to amyloid- β peptide and p3.

These observations could indicate that PS1 is the γ -secretase, although this seems unlikely because the protein has no sequence homology to known proteases. Instead, De

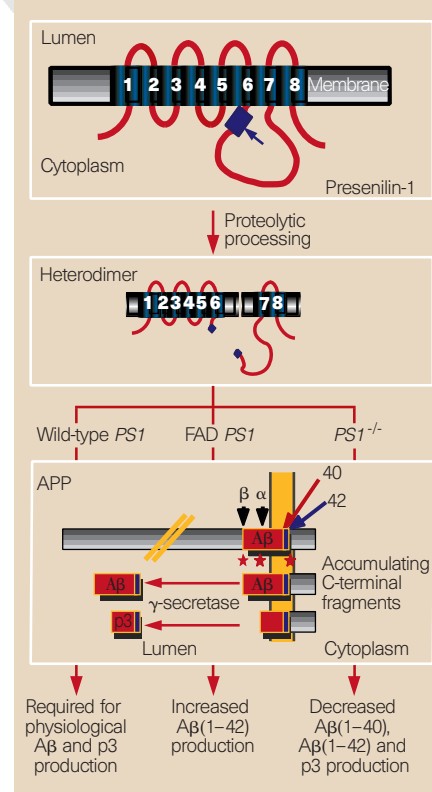


Figure 1 The influence of presenilin-1 (PS1) on proteolytic processing of the amyloid precursor protein (APP). β -secretase cleaves APP at the amino terminus of the amyloid- β ($A\beta$) peptide domain to generate a carboxy (C)-terminal fragment containing the entire amyloid- β peptide. Generation of amyloid- β peptide is decreased by α -secretase, which cleaves in the middle of the amyloid- β peptide domain to create a shorter carboxy-terminal fragment. De Strooper *et al.*¹ have found that both fragments accumulate in the PS1^{−/−} mice. The carboxy-terminal fragments are the substrate for the γ -secretase(s), which liberate amyloid- β peptide from the β -secretase-cleaved fragment, and p3 from the α -secretase-cleaved fragment. (Asterisks indicate the approximate locations of the APP mutations.)

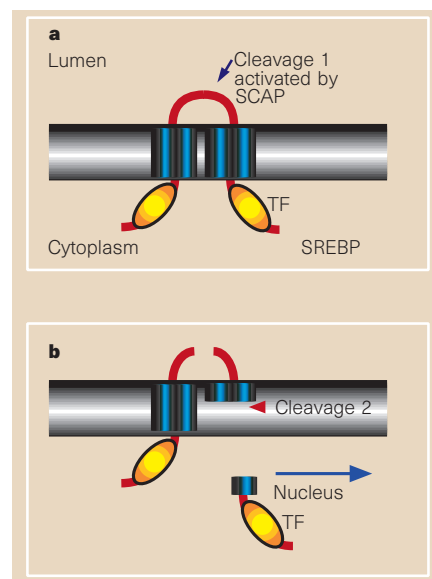


Figure 2 Model for the two-site cleavage of the sterol regulatory element binding protein (SREBP). **a**, Cleavage 1 cuts the luminal loop between the regulatory and transcriptional domains. This cleavage is somehow facilitated by the SREBP cleavage activating protein (SCAP), and it occurs in the lumen of the endoplasmic reticulum. **b**, Cleavage 2 occurs in a trans-membrane domain, liberating the membrane-bound transcription factor (TF). This cleavage is similar to the γ -secretase cleavage of APP.

Strooper *et al.*¹ discuss the exciting possibility that PS1 activates γ -secretase cleavage of APP, similar to the SREBP-cleavage activating protein (SCAP)⁸. SCAP facilitates cleavage of the sterol regulatory element binding protein (SREBP) to liberate its transcription-factor domain, which migrates to the nucleus and regulates the expression of genes involved in cholesterol biosynthesis and uptake. Cleavage of SREBP occurs in two steps (Fig. 2). Cleavage 1 cuts the luminal loop between the regulatory and transcriptional domains, and this cleavage is facilitated by SCAP. Cleavage 2 then liberates the membrane-bound transcription factor. Like the γ -secretase cut of APP, cleavage 2 occurs within the membrane.

At present, γ -secretase and the SREBP protease are the only known proteolytic activities that cleave in an environment which, according to textbook knowledge, should preclude proteolysis. There are other remarkable similarities between the two systems. Both SREBP and the presenilins are located in the endoplasmic reticulum², and the γ -secretase cleavage after amino acid 42 could occur, in part, within the endoplasmic reticulum and Golgi^{9–11}. Furthermore, the SCAP proteins are predicted to form six to eight transmembrane domains — a structure similar to that of the presenilins.

Based on these analogies, PS1 might be expected to regulate γ -secretase, and De Strooper *et al.*¹ now prove it. This physiologi-

cally normal activity is presumably disturbed by presenilin mutations, leading to increased cleavage of APP carboxy-terminal fragments after amino acid 42 of amyloid- β peptide. There is, however, one big difference between SCAP-activated SREBP proteolysis and presenilin-activated APP cleavage by γ -secretase: SCAP is currently known to activate only cleavage 1 within the lumen of the endoplasmic reticulum⁸, whereas γ -secretase cleavage of APP occurs within the transmembrane domain.

An alternative molecular explanation for the reduced production of amyloid- β peptide in *PS1*^{-/-} mice is that PS1 regulates the transport of membrane proteins from the endoplasmic reticulum to other cellular compartments. This explanation could support the observed phenotype of mutations in the *C. elegans* presenilin homologue, which could be due to altered sorting of membrane proteins within the endoplasmic reticulum. This could lead to reduced transport of proteins such as APP and the Notch receptor through the Golgi and onto the cell surface. In this case, mutant presenilins might retain APP within the endoplasmic reticulum and early Golgi, leading to increased production of amyloid- β (1–42) peptide^{9–11}. The lack of *PS1* expression would affect targeting not only of APP, but also of other membrane proteins in the endoplasmic reticulum.

Regardless of which molecular mechanism is responsible for the sharp reduction in generation of amyloid- β peptide in *PS1*^{-/-} mice, PS1 could serve as an unexpected therapeutic target for Alzheimer's disease. Inhibition of presenilin synthesis late during ageing, when the disease normally begins, would avoid elimination of the essential function of presenilin proteins during development^{5,6}. Expression of *PS1* could be repressed at the transcriptional level or by interfering with the post-translational processing of presenilin and presenilin complex formation (Fig. 1)⁴. And if levels of PS1 could be decreased enough in the brain, production of amyloid- β would be decreased and, therefore, accumulation of senile plaques could be retarded. □

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Daedalus

Crystalline clouds

Sunshine and cloud are traditional opposites. Yet droplets make up only about 10 p.p.m. of a cloud's volume — why does sunlight not go straight through? Sadly, a photon traversing a random array of droplets is almost certain to hit one sooner or later, and be scattered. So, says Daedalus, make the array non-random.

He recalls Faraday's use of iron filings to map a magnetic field. Each iron particle has a magnetic dipole induced in it in the local field direction. Axially arranged dipoles attract one another, and align themselves into chains along the lines of force. Equatorially arranged dipoles repel one another, so each chain spaces itself as far as possible from its neighbours.

The same principle must apply to electrostatic fields. A dielectric particle (such as a water droplet) must have an electric dipole induced in it by a local electric field. The dipoles will align themselves into parallel chains following that field. So, says Daedalus, put a cloud in an electric field whose lines of force point at the Sun, and its droplets will form parallel chains pointing the same way. Sunlight will pour down unimpeded between the chains, and the cloud will become almost perfectly transparent in that direction.

At first Daedalus envisaged some mighty system of captive balloons supporting charged wires, their positions constantly adjusted to keep the field pointing at the Sun. But he soon realized that a radio beam would do just as well — provided it was polarized to point its electric vector at the Sun. Even if its field reversed at many megahertz, the dipoles it induced in the droplets would still follow it perfectly.

DREADCO's weather-brightener will launch an intense radio signal into the cloud deck. It will be constantly adjusted to keep the signal polarized in the Sun's direction. A watcher anywhere beneath the cloud will see a hole in it, through which the Sun will beam down on him. The rest of the sky will still appear cloudy; yet, oddly, the landscape will be uniformly bright. The DREADCO weather-brightener will transform tourism. British seaside resorts and holiday towns will queue up to install this answer to their Mediterranean and tropical rivals. Even those rivals may install it, though for a converse reason. By aligning its signal at 90° to the solar direction, the thinnest cloud might be made opaque enough to screen out the scorching midday Sun.

David Jones

Dürer's diagnoses

The faces of the four apostles in Dürer's painting speak volumes about the saints' temperaments. This is no accident, as the artist was following the Renaissance medical theory of the four humours.

Martin Kemp

The great German painter, printmaker and theorist, Albrecht Dürer, shared with Leonardo da Vinci the quest for an art that would be 'universal' — one that constructs representations of all forms in nature on the basis of a profound understanding of natural philosophy in all its relevant facets. So, when portraying human beings, the mental and physical constitutions of each individual were to be expressed in terms of the Renaissance theories of psychology and physiology.

The medical basis for characterization was the ancient and persistent doctrine of the four humours — sanguine, phlegmatic, choleric and melancholic — which were locked into the universal, four-fold scheme of the elements, times of day, seasons, winds and ages of man.

The constitution of those who exhibited the sanguine temperament was composed from the predominance of the humour of blood, and was of the nature of air, morning, spring, Zephyr and youth. The other equations were: phlegmatics with phlegm (inevitably), water, night, winter, Auster and old age; choleric with yellow gall, fire, mid-day, summer, Eurus and maturity; and melancholics with black gall, earth, evening, autumn, Boreas and later middle age. Each temperament manifested itself not only in behaviour but also in physical characteristics and 'complexions', not least in physiognomy and the colour and texture of skin.

Dürer was fully conversant with such theories and embodied them in various prints, but their most complete expression is in the two magnificent panels of the *Four Apostles* that the ageing artist presented as his own 'memorial' to his home city of Nuremberg in 1526. Accompanied by Lutheran texts from the Bible, they testify in equal measure to Dürer's religious and intellectual allegiances.

The learned calligrapher, Johann Neudörffer, who wrote the inscriptions, recorded that each apostle embodied a specific temperament. There is the sanguine St John the Evangelist, ruddy in garment and complexion, the phlegmatic St Peter, bald and stolid, the leonine St Mark, choleric in character and physiognomy, and the sallow-skinned St Paul, who exhibits the haunted look associated with melancholy 'genius'. Like a doctor, expected to observe the external 'signs' of humoral imbalance, the spectator is presented with diagnostic clues through which Dürer invites us to discern the apostles' deepest natures in the context of the four-fold essence of God's creation.



Dürer's *Four Apostles* (Saints John, Peter, Mark and Paul), 1526 (above), and detail of heads of Mark and Paul (left).

medicine, the insights (real or imagined) are far from dead. We all act as amateur physiognomists, however often our initial reaction to someone's 'look' is confounded.

And Jerome Kagan, the Harvard developmental psychologist, in his book *Galen's Prophecy* (BasicBooks, 1997), is advocating a return to four-part division of personality — timid, bold, upbeat and melancholy — as corresponding to patterns of brain activity. □
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Embalming was used in Old Kingdom

Our knowledge of the conservation techniques used in the Old Kingdom of ancient Egypt is limited. Examinations of a clavicle fragment of the mummy of Idu II, secretary general of the pine wood trade office (2150 ± 50 BC), revealed saturation with a wealth of sodium and wood tar compounds, many of which were highly antiseptic as no microbial contamination was noticed. This pretreatment had ensured the preservation of bone alkaline phosphatase in an enzymically and immunologically active form. This extends the use of embalming to one thousand years earlier than previously thought.

In general, mummification is observed after drying of the body. To improve this process, embalming was increasingly used from the Middle Kingdom onwards. The tomb of Idu II was discovered in 1914 at Gizah and its contents were brought to the Pelizaeus Museum at Hildesheim. The mummy (inventory no. 2639) consisted of an unwrapped skeleton with bandaged head. It was soaked with paraffin immediately after recovery¹, which complicated subsequent analyses of possible ancient embalming components.

Clavicle fragments sand-blasted with Al_2O_3 were extracted three times with isooctane to remove this paraffin; gas chromatography showed no sign of 'ancient paraffin' (earthwax or similar bituminous products). The extraction was continued twice with chloroform/methanol. In this extract cyclic alcohols — including cedrol, guajacol, *tert*-octylphenol, trimethylcyclohexene methanol and octahydronaphthalene methanol — originating from the liquid fraction of wood tars and known for their bactericidal and fungicidal reactivities were found in varying concentrations. The lack of triterpenoids in the sample eliminates mastic, terebinth (Chios turpentine), frankincense and myrrh as possible sources of resin².

After evaporation of the solvent, the solid residue was re-extracted with methanol containing oxalic acid and analysed (Fig. 1a). High concentrations of diterpenoid resin acids, mainly dehydroabietic acid and its oxidized forms, and also methyl esters of these acids, were detected. This high concentration of dehydroabietic acid originates from heat-conversion of abietane- and pimarane-type resin acids^{3,4}, which are found in the balm of pine wood. Methyl esters are not abundant in natural balm resins. These compounds would have been formed during the smouldering of pine wood⁵ in the presence of excessive air. A 'smoking technique' could even have been used, as the aromatic hydrocarbons (for example, retene⁶) normally formed under

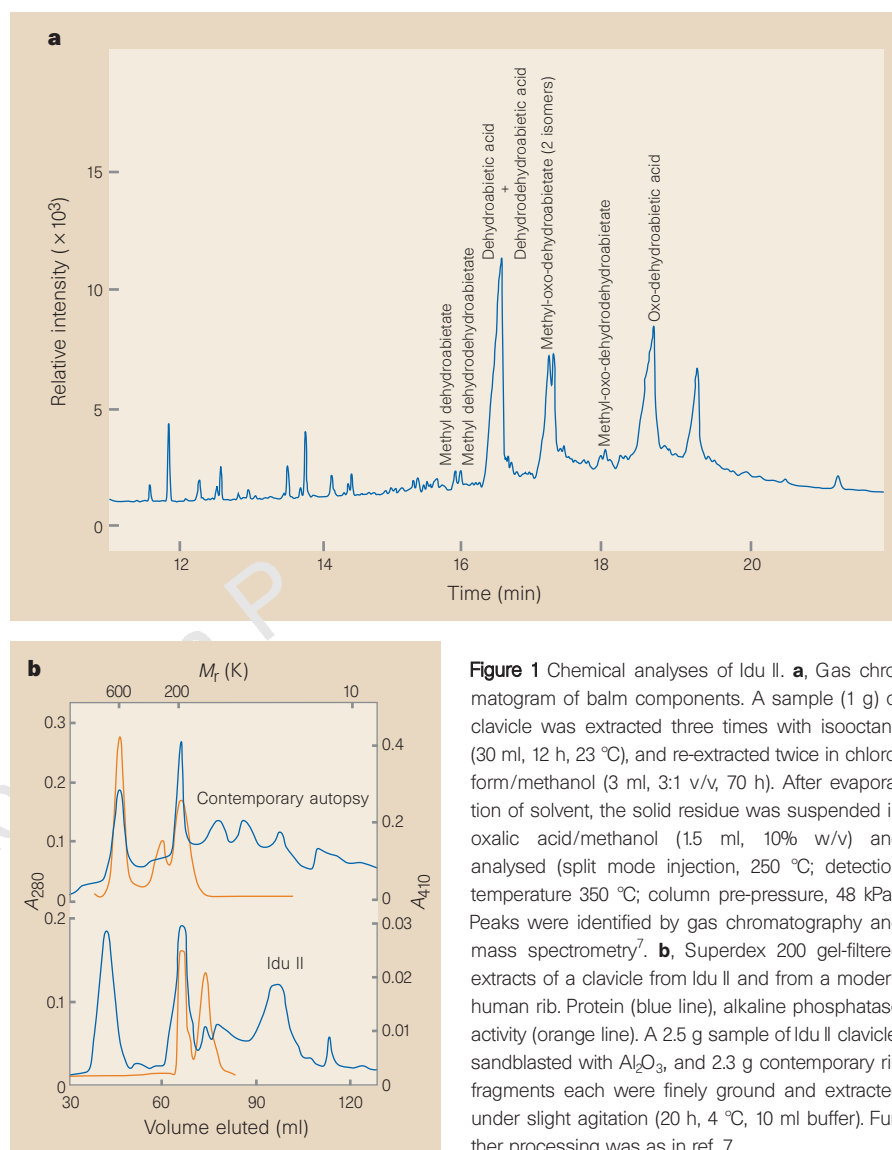


Figure 1 Chemical analyses of Idu II. **a**, Gas chromatogram of balm components. A sample (1 g) of clavicle was extracted three times with isooctane (30 ml, 12 h, 23 °C), and re-extracted twice in chloroform/methanol (3 ml, 3:1 v/v, 70 h). After evaporation of solvent, the solid residue was suspended in oxalic acid/methanol (1.5 ml, 10% w/v) and analysed (split mode injection, 250 °C; detection temperature 350 °C; column pre-pressure, 48 kPa). Peaks were identified by gas chromatography and mass spectrometry⁷. **b**, Superdex 200 gel-filtered extracts of a clavicle from Idu II and from a modern human rib. Protein (blue line), alkaline phosphatase activity (orange line). A 2.5 g sample of Idu II clavicle, sandblasted with Al_2O_3 , and 2.3 g contemporary rib fragments each were finely ground and extracted under slight agitation (20 h, 4 °C, 10 ml buffer). Further processing was as in ref. 7.

oxygen deficiency are absent. We conclude that the bones had been soaked with this balm, reopening the discussion about whether or not Idu's body was defleshed in whole or in part before embalming. Further support for the suggested conservation of the skeleton was expected from sodium analyses. The 12-fold higher sodium content ($4,100 \mu\text{mol per g dry weight (d.w.)}$) in Idu's clavicle, measured by atomic emission spectrometry, compared with sodium analyses of bones from modern autopsies ($330 \mu\text{mol per g d.w.}$), suggests that natron was applied directly to the skeleton.

The successful isolation of ancient alkaline phosphatase from a ptolemaic mummy⁷ prompted us to examine the enzyme's preservation in Idu. Clavicle fragments were used for isolating the enzyme. During gel chromatography, the elution profile was essentially the same as that

observed for the extraction of a modern bone sample (Fig. 1b). Two peaks of enzyme activity for proteins of relative molecular mass (M_r) around $(230 \pm 30) \times 10^3$ were seen, with an additional peak at M_r $(115 \pm 15) \times 10^3$ in the modern sample. This protein peak is consistent with the polypeptide frame of the bone enzyme, which is composed of two subunits at M_r 57.2×10^3 (ref. 8). The enzyme activity was improved after affinity chromatography when the specific activity rose to 200 mU mg^{-1} , ~20% of the activity obtained with a contemporary enzyme.

The reactivity of the mummified enzyme was inhibited by 80% in the presence of 1,10-phenanthroline; activity was restored after the addition of Zn(II) . L-Homoarginine, an allosteric inhibitor of the bone enzyme⁹, diminished the activity by 77%. A monoclonal antibody was applied on to the lectin-

chromatographed enzymically active fraction¹⁰. There was an unequivocal immunoreactivity of 23% compared with that of the enzyme obtained from modern autopsy. Furthermore, no microbial contamination of the bones was detectable⁷.

The wealth of pine wood compounds and sodium in the bones support the proposal that IDU II has been defleshed or skeletonized at least in part before embalming¹. Desiccation with natron and embalming was thus being practised at least one thousand years earlier than previously thought. This 4,000-year-old conservation has been most beneficial for preserving the functional and structural intactness of bone alkaline phosphatase.

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What can stochastic resonance do?

Stochastic resonance^{1–3} is often defined as a noise-induced rise (and then fall, for higher noise intensities) of the signal-to-noise ratio (SNR) of a weak narrow-band signal in a nonlinear system. Various applications of this phenomenon are being explored, in particular the possibility that stochastic resonance might help enable biological cells to respond to weak 50–60-Hz electromagnetic fields, far below the thermal noise level^{4,5}. We therefore feel that its place within the broader physics context should be specified more clearly. Specifically, what stochastic resonance can, and cannot, be expected to do.

The idea of stochastic resonance might seem counterintuitive. However, soon after its discovery in bistable systems, it was realized that the idea amounted to a fairly straightforward extension of earlier work by Debye⁶ on reorienting polar molecules, and

that the occurrence of stochastic resonance in the general case could be treated⁷ with a traditional technique of statistical physics⁸: linear response theory (LRT). It is well known in the LRT context that the response of a system to signals in certain frequency ranges can be strongly increased by noise, for example by raising the temperature. Examples range from currents in electron tubes to optical absorption near absorption edges in semiconductors. The threshold-less model considered by Bezrukov and Vodyanov⁹, the subject of recent discussions^{4,5}, displays noise-induced increases in both the signal and the SNR. Their formula for the SNR represents a special case of the general LRT expression given earlier⁷ and subsequently applied to many different systems^{7,10–12}.

An important consequence^{10,13} of LRT, relevant to the recent discussion^{4,5}, is that, for a system driven by a signal and Gaussian noise, the SNR at the output, R_{out} , does not exceed that at the input, R_{in} . For a linear system $R_{out} = R_{in}$, and the SNR decreases with increasing noise intensity. For a nonlinear system R_{out}/R_{in} can be small; then the provision of additional noise can sometimes help to increase the SNR at the output, back towards its value at the input. This latter effect constitutes stochastic resonance. Quite independently of parameter choice^{4,5}, therefore, the SNR of the 50–60-Hz signal inside the biological cell (output signal) cannot be expected to exceed that of the external signal coming from the environment (input signal).

Stochastic resonance can decrease quite markedly the SNR degradation of a noisy signal caused by its transduction through a nonlinear element, but it does not provide a mechanism by which the SNR of the weak input signal can meaningfully be enhanced.

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CCR5 is characteristic of Th1 lymphocytes

CD4⁺ lymphocytes can be assigned to two subsets¹. Th1 lymphocytes secrete interferon gamma (IFN γ) and lymphotoxin, promoting cell-mediated immunity to intracellular pathogens; and Th2 lymphocytes secrete interleukins 4 and 5 (IL-4 and IL-5), which function in allergy and humoral immunity to parasites. Th2 lymphocytes preferentially express the chemokine receptor CCR3 (refs 2,3). We have studied the occurrence of two additional chemokine receptors, CCR5 and CXCR3, in human, antigen-specific CD4⁺ Th1 and Th2 cell clones⁴.

CCR5 was expressed at high levels in Th1 and was virtually absent from Th2 lymphocytes; CCR3 was undetectable in Th1 and moderately expressed in Th2 cells (Fig. 1a, c), but both were highly positive for CXCR3. When we assessed receptor function by measuring chemotaxis in response to selective chemokines⁵, Th1 lymphocytes responded to MIP-1 β and IP10 but not to eotaxin, and Th2 lymphocytes responded to eotaxin and IP10 but not to MIP-1 β (Fig. 1b, d), as expected from the receptor expression data. Analysis of a panel of T-cell clones⁴ confirms that CCR5 is characteristic of the Th1 phenotype, being expressed at high levels in nine of nine Th1 clones. Only one of nine Th2 clones was positive for CCR5. Most Th2 clones expressed moderate to low levels of CCR3, responding accordingly to eotaxin. The presence of CXCR3 was detected in all clones tested, but expression and chemotaxis were higher in Th1 clones.

Flow cytometry and function analysis were performed on naive cord-blood T lymphocytes, polarized towards the Th1 phenotype⁶. Whereas naive cord-blood T lymphocytes were negative, Th1-polarized cells expressed moderate levels of CCR5 and high levels of CXCR3 but were CCR3-negative, and migrated towards MIP-1 β and IP10 but not towards eotaxin (Fig. 1e, f).

Receptor expression and function were studied in T lymphocytes from rheumatoid joints, which acquire the Th1 phenotype *in vivo*⁷. T-cell areas of the rheumatoid synovium, defined by staining with anti-CD3, showed high levels of CCR5 and CXCR3 and only borderline staining for CCR3 (Fig. 2). The same staining pattern occurred in the characteristic lymphocytic infiltrates around microvessels (Fig. 2, insets).

In addition, T lymphocytes recovered from the synovial fluid of rheumatoid joints, which exhibit a Th1 phenotype, expressed high levels of CCR5 and CXCR3 and low levels of CCR3, and were responsive to MIP-1 β and IP10.

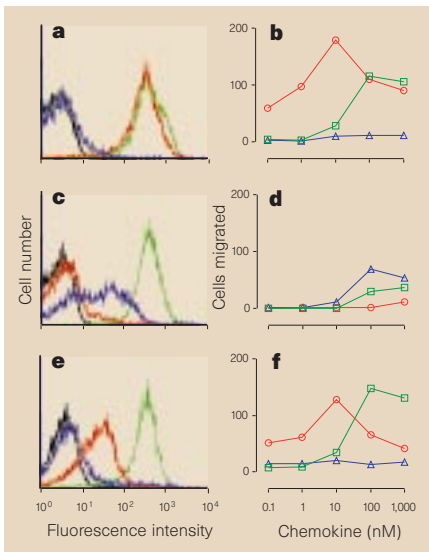


Figure 1 CCR5 is preferentially expressed in Th1 lymphocytes. Flow cytometric analysis of CCR5 (red), CXCR3 (green) and CCR3 (blue) expression and chemotaxis in response to MIP-1β (red), IP10 (green) and eotaxin (blue) is shown in Th1 clones (a,b), Th2 clones (c,d) and Th1-polarized cord blood cells (e,f). Isotype-matched control immunoglobulins are shown in black. Cloned Th1 and Th2 lymphocytes were re-stimulated with phytohaemagglutinin in the presence of irradiated feeder cells and cultured for 10–12 days⁶. Cord blood cells were subjected to two rounds of polarization towards Th1 with IL-12 and anti-IL-4 (ref. 6). For flow cytometry, cells were stained with anti-CCR5 (5C7) (ref. 8), anti-CXCR3 (1C6.2) (ref. 13), anti-CCR3 (7B11) (ref. 14) and control IgG followed by phycoerythrin-conjugated goat anti-mouse IgG. Chemotaxis was performed as described¹⁰. Cells were counted in five randomly selected fields at a magnification of $\times 1,000$.

The study adds weight to the notion that chemokine receptors are expressed in T lymphocytes depending on their state of activation or differentiation⁵. Naive and memory T lymphocytes do not respond to chemokines that are frequently produced in inflammation. The expression of CCR1, CCR2, CCR5 and CXCR3 and chemotactic migration depends on activation, in particular with IL-2 (refs 8–11). In contrast, CXCR4 is present and functional in resting and stimulated T lymphocytes⁵.

In T-cell lines generated from human donors by culture with IL-2 (ref. 10), we observed a marked variation in CCR5 expression and responsiveness to MIP-1β between donors. Like CCR1 and CCR2 (ref. 10), CCR5 is rapidly lost in the absence of IL-2, and activation by anti-CD3 and anti-CD28 antibodies results in CCR5 downregulation and loss of migration (not shown). CXCR3 is present on Th1 and Th2 cells and on T cell lines that are cultured with IL-2, whereas CCR3 is restricted to Th2 cells⁵. Like CCR1, CCR2 and CCR5, CCR3 is downregulated on T-cell

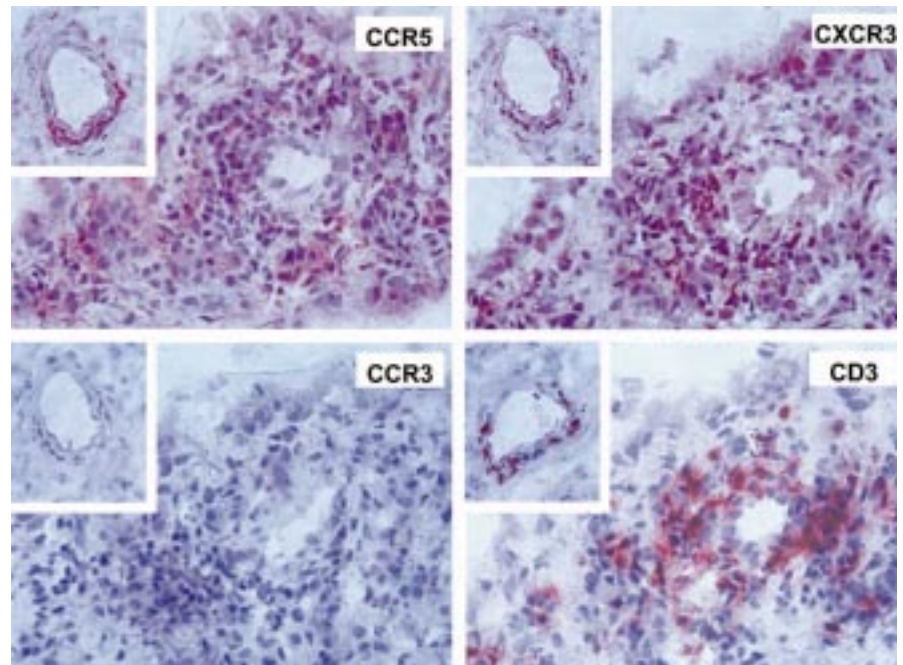


Figure 2 Expression of CCR5, CXCR3 and CCR3 in rheumatoid arthritis. Immunohistochemical analysis of serial sections of synovial tissue from rheumatoid arthritis patients stained for CCR5, CXCR3, CCR3 and CD3 (ref. 3). Cryostat sections were fixed and stained with anti-CCR5 (5C7) (ref. 8), anti-CXCR3 (1C6.2) (ref. 13), anti-CCR3 (7B11) (ref. 14) or anti-CD3 followed by biotin-labelled sheep anti-mouse antibodies and streptavidin-biotin-alkaline phosphatase. The colour reaction was developed with New Fuchsin containing levamisole. The slides were counterstained with Mayer's haematoxylin. Representative fields are shown. Insets show perivascular infiltration of CCR5-, CXCR3- and CD3-positive cells.

activation². Levels detected in this study were moderate and variable; thus CCR3-bearing lymphocytes might constitute a Th2 subpopulation. This receptor is characteristic of T lymphocytes recruited into eosinophil-rich sites of allergic inflammation³. Chemokine receptor expression could constitute a major regulatory element for the composition of the lymphocytic infiltrates in different types of inflammatory pathology. In fact, Th2 lymphocytes bearing CCR3 are frequent in allergic infiltrates³, whereas CCR5- and CXCR3-positive Th1 lymphocytes predominate in the rheumatoid synovium.

The recruitment of CCR5-positive CD4⁺ T cells to sites of inflammation and immune reactions might contribute to the spreading of monocytotropic HIV-1 strains that use CCR5 as co-receptor. This suggestion is in agreement with the observation that in HIV-positive individuals microbial infections, which induce a Th1 response, are followed by a burst in viraemia¹².

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Straining the prion hypothesis

Aguzzi and Weissmann in their News and Views feature¹ correctly state that research on the molecular genetics of PrP protein has contributed greatly to our knowledge of the transmissible spongiform encephalopathies (TSEs). But their firm belief that these diseases are caused by rogue proteins ('prions') leads them to misrepresent alternative hypotheses of the nature of the agent, dismissing all non-believers as "the die-hard pro-virus faction"¹. In fact, the prion hypothesis is far from proven:

the precise nature of a prion still eludes identification and the prion hypothesis has yet to explain satisfactorily the many strains of TSE². The alternative 'virino' hypothesis is not a conventional virus hypothesis, but it addresses the diversity of biological properties of the TSEs². It proposes an agent-specific replicable informational molecule, yet to be identified, bound to a protective host protein, PrP.

In most TSEs, infectivity and a disease-associated, conformationally altered form of PrP (PrP^{Sc}) first accumulates in lymphoreticular and secretory organs³. Infection then spreads to the nervous system, leading eventually to neurodegeneration, accompanied by widespread accumulation of PrP^{Sc}. The time-course of the disease and of the pathology in the brain⁴ is under precise control, regulated by both the strain of the infecting TSE agent and the host genes, fundamentally but not solely by the gene which encodes PrP (ref. 2).

The normal function of PrP protein remains unknown and its roles in disease are disputed. The prion hypothesis is based on the structural properties of PrP, and proposes that an abnormal form (PrP^{Sc}) becomes infectious^{5,6}. But, although infection tends to copurify with PrP^{Sc}, not all PrP^{Sc} is associated with infectivity⁷, and so a conformationally altered subfraction of PrP (named either PrP* (ref. 1) or a redefined PrP^{Sc} (ref. 6)) is now proposed, but has yet to be testably defined. Yet the term 'prion' is being used widely and prematurely as a synonym both for the causal agent and for PrP protein, as well as for the postulated structure in the protein-only hypothesis.

The function of PrP in TSEs was originally predicted from the properties of the mouse gene *Sinc*, which encodes two alleles of PrP (ref. 8) and determines the incubation periods of TSEs in mice⁹. The replication site hypothesis⁹, on which the virino hypothesis is based, predicted that PrP interacts directly with the agent to "determine the crucial rate-limiting step in pathogenesis"¹⁰. Because incubation periods in PrP heterozygote mice are longer than in either homozygote with some agent strains, it was reasoned that the replication site⁹ comprises PrP multimers (possibly dimers¹⁰) which interact with the agent. This led to several predictions and implications which have been fulfilled by experimental observation (Table 1). The results neatly conform to the hypothesis that the agent interacts with multimeric PrP. But the prion hypothesis requires an extra host factor, 'protein X'⁶, to explain these findings.

TSEs exist as many different strains that carry information encoding their own biological properties. The strain-specific information of the infecting agent inter-

Table 1 Experimental tests of the replication site hypothesis

Prediction/implication	Experiment
A limited number of replication sites exist	Surgical or genetic splenectomy lengthens incubation period ^{15,18}
There is competition between scrapie strains	Prior infection with a TSE strain with a 'long' incubation period prevents or slows the development of disease from a strain with a 'short' incubation period ¹⁹
Increasing (decreasing) the number of PrP genes transgenically should increase (decrease) the number of replication sites and shorten (lengthen) incubation periods	1. PrP-null mice (functional deletion of the gene for PrP) do not replicate TSE infectivity 2. Mice with one copy of the gene for PrP have longer incubation periods than normal (2 copy) mice ²⁰ 3. Incubation period in multicopy PrP homozygotes is inversely proportional to copy number
Interspecies heteromers could form between the transgene and the endogenous gene products to profoundly affect incubation periods, sometimes overriding the effect of adding more replication sites	1. There is a much longer incubation period in the transgenic animals with approximately two copies of the hamster and two endogenous mouse genes, instead of the short incubation periods normally found with the 263K strain of scrapie in hamsters ²¹ 2. Incubation periods as short or shorter than the 'real' hamster incubation period are obtained when much larger numbers of the hamster gene are incorporated ²¹ or the mice are crossed with PrP-null mice to remove the mouse PrP gene ²⁰

acts with host PrP genotype to determine the incubation period², and specifies brain targeting of lesions (such as vacuolation and PrP accumulation)⁴ and PrP^{Sc} glycoform profile¹¹. An individual host can be infected with more than one TSE strain. Different TSE strains specify different disease outcomes in hosts with identical primary structure of PrP (ref. 2). Further, the same strain, for example the bovine spongiform encephalopathy agent, can retain its biological characteristics after passing through many different species, including humans¹², which have different PrP proteins.

No mechanism has yet been proposed that can satisfactorily explain how PrP protein alone could specify and retain multifactorial TSE strain characteristics. On the other hand, the virino hypothesis^{7,10,13-15} proposes a small host-independent informational molecule encoding strain-specific information which is bound to and protected by a host protein, PrP. This fulfils the requirements of all the biological experimental evidence obtained and is compatible with the biophysical and biochemical data⁷.

For example, the small target size of the agent calculated from ionizing irradiation studies has been reconciled with the size of nucleic acid genomes in small viruses¹⁶. The virino informational molecule is expected to be an untranslated nucleic acid^{13,14} simply because nucleic acid is the only replicating biological molecule identified so far to carry discrete genetic information. Any other host-independent information-carrying molecules that existed would fit the hypothesis.

The molecular structure of the agent is still to be determined, but there is now enough evidence to formulate testable, falsifiable predictions from hypotheses. For example, a clear demonstration of *de*

novo production of infectivity¹⁷ through defined conformational changes of PrP would undermine proposals of a host-independent informational molecule which 'hard codes' the agent's genetic information.

The discovery of an informational molecule with strain-specific properties, for example a nucleic acid, would refute a PrP protein-only hypothesis. Until the matter is settled, it should be recognized that there is more to comprehending the biological diversity of TSEs than 'prion' protein.

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Instruments and the imagination

Image and Logic: A Material Culture of Microphysics

by Peter Galison

University of Chicago Press: 1997. Pp.955.
\$90, £71.95 (hbk); \$34.95, £27.95 (pbk)

S. S. Schweber

The experimenter... is not one person, but a composite.... He is a social phenomenon, varied in form and impossible to define precisely. One thing, however, he certainly is not. He is not the traditional image of a cloistered scientist working in isolation at the laboratory bench. (Alan Thorndike, quoted in *Image and Logic*)

At the end of the nineteenth century, physics was transformed. The atomic and subatomic world — the realm of the invisible — became the focus of much of the enterprise, and reductionist explanations in terms of the atomic and molecular constituents of matter began to assume a privileged role.

By the early 1920s, visions of unification — at the time, of physics and chemistry — and the belief that the phenomena of nature could be described by a limited number of elementary constituents, the dynamics and interactions of which could be represented by an ultimate, ‘fundamental’ theory, became part of the metaphysics of physics. The formulation of quantum mechanics in the mid-1920s, and its amazing empirical and instrumental successes, further bolstered reductionism and the drive towards a unified description of the forces of nature.

It had been usual until the early 1980s to tell the story of the advances of physics in the twentieth century as a grand narrative beginning with Max Planck and Albert Einstein and culminating with the formulation of the Standard Model of electro-weak and -strong interactions in the 1970s. Theoretical understanding took pride of place in the story, and the commitment to reductionism and unification was seen as an important factor in explaining the success of the programme. The Kuhnian model of the growth of scientific knowledge, with its revolutionary paradigm shifts, buttressed the primacy of theory and the view that experimentation and instrumentation were subordinate to, and entrained by, theory.

The situation changed after Ian Hacking, Peter Galison, Bruno Latour, Simon Schaffer and other historians, philosophers and sociologists of science reanalysed and reassessed the practice and role of experimentation. It became clear that accounting for the growth of knowledge in the physical sciences during the twentieth century is more complex than had previously been believed.

In his splendid book, *Image and Logic*,

Galison offers a framework for understanding what physics is about in the twentieth century. He makes a convincing case for regarding experimentation, instrumentation and theory as quasi-autonomous subcultures with languages and practices that are distinct, yet linked and coordinated. Experimental, theoretical and instrumental practices do not all change of a piece: each has its own periodicity, and their relation to one another varies depending on the specific historical situation in which each is embedded. One of Galison’s aims is to demonstrate the deep continuity of experimental practices across theoretical and instrumental breaks, and he does this through an analysis of the instruments of modern microphysics that make the invisible both objective and believable.

Galison’s focus is on detectors, the instruments, large and small, that were and are the mediators between the production of phenomena and the production of evidence. He identifies and describes the two competing traditions of instrument-making in the field. The image tradition — starting with C. T. R. Wilson’s cloud chamber and its progenies of the 1920s and 1930s, continuing with the nuclear emulsions of the 1940s and 1950s, and culminating with the bubble chambers of the 1950s and 1960s — aspired to produce

representations of natural processes in all their richness and complexity.

The aim of all the various forms of image-making in that tradition was the production of pictures of such clarity that a single picture would serve as evidence for a new particle or effect. In the logic tradition the instruments were electronic counters that were aggregated and embedded in a matrix of electronic logic circuitry to capture desired events. The first prototypes were the Geiger–Müller, scintillation and Cerenkov counters, the progenitors of the spark and wire chambers. These are counting — rather than image-making — devices that gather masses of data to make statistical arguments for the existence of a particle or an effect.

In the early 1970s, a symbiosis of the two traditions took place, resulting in electronic bubble chambers, which are detectors that produced computer-generated pictures using data generated by wire chambers. These advances were made possible by the revolution in microelectronic circuitry and the newly developed charge-coupled devices.

At the same time the successful operation of storage rings made experiments with colliding beams standard practice. In a conventional accelerator, the collision products in a fixed-target experiment travel primarily in the forward direction. With colliding beams,



Travels with a camera

Photographer William Neill lives in Yosemite National Park and has published many pictures of that part of California. But his latest portfolio, *Landscapes of the Spirit* (Bulfinch, \$40), takes the reader on a tour of other parts of North America, and includes some breathtakingly beautiful images. *Twilight Surf* (above) was taken on the Big

Sur coast of California. Neill writes: “The five-minute exposure necessary to make this image turned pounding surf into poetic fog. By the time the exposure was over, I barely had enough light to find my way back up the cliffs. I used a graduated neutral-density filter to balance the brightness of the sky with the much darker surf.”

the collision products spew out in all directions, hence the requirement for larger, more complex, multicomponent 4π detectors. These multicomponent electronic detectors, such as the Mark I and the time projection chamber, introduced a further change of scale, in the physical size of the detector, in the number and kinds of people involved and, most importantly, in the role of the computer and of computing. These transformations altered both the definition of the experimenter and the structure of demonstration, and brought about a re-evaluation of what it meant to be an author. Galison characterizes this shift from pure to hybrid devices as a transition from the 'modern' to the 'postmodern'.

In his introduction Galison indicates that the framing questions for his book were: "Who counts as an experimenter?" and "What counts as an experiment?". *Image and Logic* establishes how the answers to these questions changed over the century as accelerators increased in energy, as the site where the experiments are carried out was transformed, as engineers took over the function of running the accelerators, as computers assumed an ever more central role in running the operation and in interpreting the data, and as the nature of the interactions between the actors that make experimentation possible — experimenters, engineers, theorists, computer programmers, calorimeters, computers and so on — changed.

But the book is much more than that. It offers a detailed, valuable account of what made possible the developments in experimental high-energy physics. Galison examines not only the general context, but also probes deeply into the lives of institutions and individuals. We obtain accounts of how universities, instrumentation and the management of scientific activities changed during the Second World War. We are given sensitive portraits of, among others, Charles T. R. Wilson, Marietta Blau, Cecil Powell, Donald Glaser, Luis Alvarez, Stanislaw Ulam and David Nygren, individuals who, by their innovations, were responsible for radically transforming the enterprise of high-energy physics.

We are also shown the cunning of reason at work: Glaser inventing a table-top ether-filled bubble chamber in the hope of salvaging the practices of cosmic-ray physics in which a single experimenter can be responsible for and in control of all aspects of an experiment, only to see the instrument transformed in Alvarez's hands into a very sizeable hydrogen-filled bubble chamber. Because this detector was ideal for the new accelerators coming on line, it became the centrepiece of an industrial-scale operation involving cryogenic and electronic engineers, and teams of scanners. It was able to produce such a huge mass of data that it required the automation of the scanning process. This in turn entrained a scientific

way of life, in which data analysis essentially became the experiment, totally at odds with Alvarez's original vision. Eventually Alvarez himself left the field, finding its gargantuan aspects unpalatable.

The chapter on the time projection chamber describes what is involved in the design and construction of large, heterogeneous pieces of apparatus. Galison reports what it takes to carry out successfully projects in which the level of complexity makes it impossible for any one individual to master all the technical details; where consequently no 'one' is in charge and a collaborative style of interaction must be implemented, and where seemingly it is the computer and its languages that holds the enterprise together. Galison melds the technical factors, the managerial aspects and the interpersonal relationships into a seamless narrative that conveys both the drama and the impressive achievement of the project.

Galison has an ability to synthesize that overwhelms. The chapter on 'artificial reality' is a wonderful introduction to the art and science of simulation and modelling, to Monte Carlo methods and the genesis of these practices in the design of fission and fusion weapons, and to the way in which the simulation has become a subdiscipline in many areas of science and has transformed its practice in these areas. In high-energy physics it has generated a new kind of phenomenology that blurs the line between experiment and theory. The careful reader will also find out how a geometrical interpretation of logic was derived by Ulam from his computing activities, and many other such interesting insights.

As should be clear from the above, I consider *Image and Logic* to be a magnificent achievement. As a penetrating history of experimental high-energy physics during the twentieth century, it will be of great interest to physicists, historians, sociologists and philosophers of science. But *Image and Logic* is more than that. It is a brief for 'mesoscopic history': for writing history at a level between macroscopic, universalizing history and microscopic, nominalistic history.

Galison proposes treating the movement of ideas, objects and practices as one of local coordination — both social and epistemic — made possible through the establishment of pidgins and creoles. For Galison these 'interlanguages' become a central decentred metaphor. In the case of physics, the picture that Galison paints is that of separate but correlated subcultures bound and stabilized by these interlanguages. Galison's plea for this new historiography will be widely discussed, for his book will surely be very influential and generative. □

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Thinking machines

The Cambridge Quintet: A Work of Scientific Speculation

by John L. Casti.

Little, Brown/Addison-Wesley: 1988. Pp. 181. £16.99, \$23. US publication date, April 1998

Goodbye Descartes: The End of Logic and the Search for a New Cosmology of the Mind

by Keith Devlin

Wiley: 1997. Pp. 301. \$27.95, £17.99

Igor Aleksander

John L. Casti's *The Cambridge Quintet* ends with a languid "The thinking machine conundrum is not going to be solved in one evening". Both this book and Keith Devlin's *Goodbye Descartes* give the impression that the conundrum continues to defy 'solution', but this need not detract from the enjoyment of reading these works, each of which has a novel way of approaching the discussion.

Casti uses the device of a fictional dinner party in Cambridge set around the middle of this century. C. P. Snow is the host. His task as a civil servant is to advise the government on



Snow.

the feasibility or otherwise of building a machine that "thinks like a human being".

The four guests, with much literary licence, represent the main pillars of the debate. Alan Turing is there, issuing the mathematical challenge that universal computation machines, being universal, must be capable of thought. Erwin Schrödinger, as a confident physicist, believes that thought in living objects and machines may be subject to the same laws of physics. The third diner is J. B. S. Haldane who, in his role as a geneticist, argues that the biological and evolutionary make-up of living beings gives them a character that cannot be emulated on computers. The last guest is Ludwig Wittgenstein, who will have none of it.



Wittgenstein.

For him the idea of a thinking machine is an absurdity, an oxymoron. Casti's conversational exposure of these extreme views, which are widely held today, suggests that they may be simply irreconcilable.

With greater hope, Devlin holds that 'thought' has a character that transcends mathematics, particularly logic. This is not in the same spirit as those who believe that science is not yet sufficiently developed for the task, as Roger Penrose argued in *Shadows of the Mind* (Oxford University Press, 1994); rather, Devlin argues that mathematics is too advanced, too precise, to

match the fluidity of human thought. This gives him the opportunity of writing with great clarity on logic and its useful but limited role in computation. Devlin concludes that a 'soft' form of mathematics might do the trick. Unfortunately we never find out much about it: as Devlin himself admits, he is only hoping this might be so.

Despite the qualities of these books, they may be too ready to accept the lack of progress on the 'thinking machine' question posed by Turing more than 60 years ago. Are



Haldane.

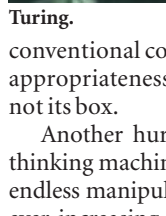
the hurdles better understood now? The first barrier to progress comes from the word 'machine' in the context of 'thinking'. It is generally assumed that such a machine would be a conventional computer which, when given an appropriate program, would behave in a way akin to a thinking human. Unfortunately much of the argument comes to a halt with the functional emptiness of this model: the computer itself does nothing; some programmer must work out every step of what the machine is to do.



Schrödinger.

By contrast, the brain is clearly a functional machine of a kind, as it does not rely on anything like a program. It is beautifully structured to be driven by both evolved structure and learned function, neither of which may be available to a programmer. Understanding how this mechanism contributes to the experience of sensation and thought is top of the current agenda.

It helps that evolution and learning can be modelled and analysed in an appropriate artificial domain: that of neural systems. Parallelism, adaptation and modularity are needed to make real machines work in emergent, seemingly intelligent ways. Models need these self-organizing features. That the modelling is often done on a conventional computer is a red herring; the appropriateness of the model is important, not its box.



Turing.

Another hurdle is the assumption that thinking machines will work by performing endless manipulations of simple symbols at ever-increasing speeds. The symbols of a neural machine, whether real or artificial, are neural firing patterns which are wonderfully extravagant, rich, diverse and sufficiently expressive to be capable of directly supporting subtle sensations that could distinguish between the thought of a good Burgundy and plonk, or between loving and liking. The

notion of the brain as a manipulator of parsimonious symbols or, indeed, as some kind of data-processing machine is a customary but sterile starting point for a science of the mechanics of thought.

Although both Casti and Devlin hint at the possibility of evolving and adaptive machines, they largely avoid the topic. Perhaps they do not wish to offend the many who, over the past half century, have believed in the supremacy of an empty computing box that begs for someone to tell it what thinking is. □

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Change of mind

Molecules of Emotion: Why You Feel the Way You Feel

by Candace B. Pert
Scribner: 1997. Pp. 368. \$25

Samuel H. Barondes

In 1970 Candace Pert was admitted to Johns Hopkins University as a graduate student in pharmacology, and soon began doctoral research in the laboratory of Solomon Snyder. By autumn 1972 she had helped to develop the first practical binding assay for opiate receptors in brain homogenates, and this became the basis for studying the

interaction of these receptors with drugs and natural ligands.

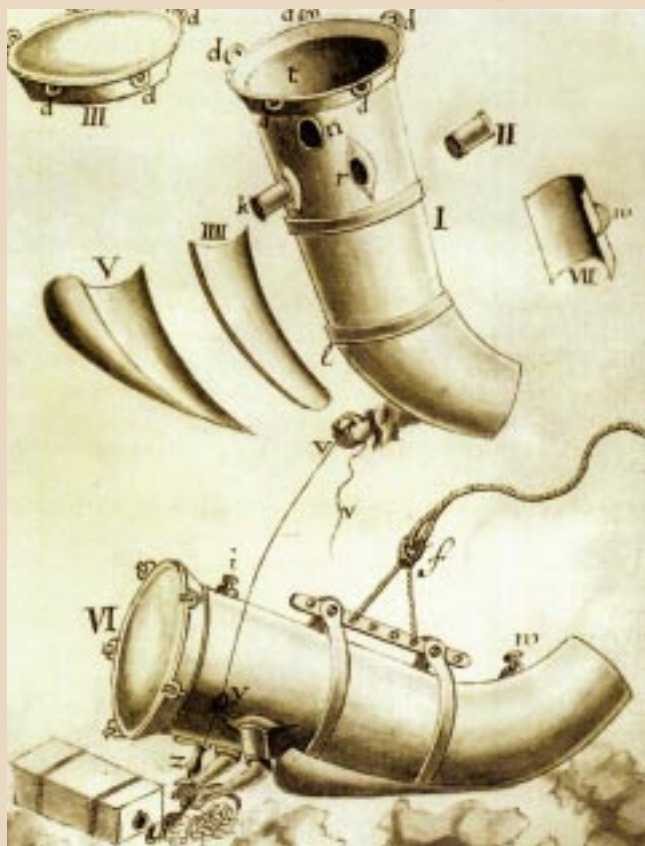
Three years later her work with Snyder had earned her an independent position at the 'Palace' (her name for the National Institutes of Health in Bethesda, Maryland), where she remained until 1987, publishing more than 200 scientific papers, many of which were very widely cited.

In 1978 Pert faced a great personal crisis when she learned that Snyder would receive the Lasker Award for work on opiate receptors, along with John Hughes and Hans Kosterlitz, who had determined the structures of two endogenous opiate peptides, the enkephalins. Believing she should have been included among the winners, Pert made a formal protest that became public and was recounted in many places, including Robert Kanigel's *Apprentice to Genius* (1986) and Jeff Goldberg's *Anatomy of a Scientific Discovery* (1988). Now, in *Molecules of Emotion*, Pert retells the story from her point of view, and lets us know what she has been doing since then.

Despite its subtitle, this book is essentially a memoir. Interspersed throughout the first half are some discussions about neuro-peptides (Pert's 'molecules of emotion'), though even the endogenous opiate peptides are considered in only a cursory way, and there is no mention of a multitude of discoveries about the structure and biology of opiate receptors that followed from Pert's

Hidden depths of underwater salvage

Captain Jacob Rowe's patented diving engine was the highlight of a remarkable salvage operation to recover 17 chests of specie from the Dutch East Indiaman *Adelaar*, wrecked off Barra in the Outer Hebrides, Scotland, in 1728. The machine could be used — albeit with great discomfort — at depths of up to 18 metres. The *Encyclopedia of Underwater and Maritime Archaeology* edited by James P. Delgado (Yale University Press, \$55) is a treasure-trove of information about the submerged past. Its 500 pages describe sites around the world, the techniques and tools of underwater archaeologists, ethical and legal aspects, and important institutions and individuals.



seminal work.

Molecules of Emotion is mostly about Pert's life in science, which she often discusses with startling candour. She recounts, for example, how, late in 1975, she and a collaborator betrayed a confidence by obtaining the then unpublished structure of enkephalin "from a secret source", made the synthetic peptide, and showed that it had an analgesic effect when injected in the brain, aiming "to get it printed as close on the heels of Hughes's paper as possible". With remarkable openness she traces her hunt for a treatment for AIDS to a voice she heard in 1985 "echoing inside my own head! It was a strong male voice that commanded: 'You should do this!'".

Following "the direction [that] had been dictated by a voice in my head while I stood at a podium in Maui", Pert enlisted others to help her to design peptides that might block the attachment of an HIV envelope protein to a receptor on T cells using a receptor-binding assay.

She quickly found a synthetic peptide — peptide T (for threonine) — that seemed to work. It so interested the "Second Biggest Drug Company on the Planet" that its representatives persuaded her to give up her tenured position at the National Institutes of Health for a new laboratory they built for her, called "Peptide Design". But in little more than a year their support for this laboratory was terminated, and "Peptide Design became Peptide Demise".

Devastated by this turn of events, and disheartened by the hostility with which her work on peptide T was received by others studying HIV, Pert decided to withdraw from the intensely competitive arena of science, and to devote herself to the "new modalities of personal healing". She was soon "earning a reputation... as a 'body-mind' scientist", and the warm welcome she received when she spoke at the 1991 meeting of the American Association of Holistic Medicine made her "feel totally at home with the new-paradigm crowd".

Towards the end of the book she offers opinions and advice based on what she has learned about holistic medicine, in a section called "Lifestyles of the Healthy, Whole and Conscious: an Eight-Part Program". There is also an appendix: "Prevention-Oriented Tips for Healthful, Blissful Living".

This is not a run-of-the-mill autobiography of a scientist. As Pert says at the outset: "Perhaps my journey, intellectual as well as spiritual, can help other people on their paths". *Molecules of Emotion* can certainly serve that purpose, but the lessons drawn from it by many readers may be rather different from those drawn by Pert herself. □

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Worried to death?

Culture of Fear

by Frank Furedi

Cassell: 1997. Pp. 224. £45, \$70 (hbk); £11.99, \$19.95 (pbk)

Haunted Housing: How Toxic Scare Stories Are Spooking the Public Out of House and Home

by Cassandra Chrones Moore

Cato Institute: 1997. Pp. 274. \$21.95 (hbk); \$11.95 (pbk)

John Galloway

"All life," Sam the Gonoph concluded in *A Nice Price* by Damon Runyon, "is six to five against". Of course, in the end we will all be dead. However, in the meantime we would like the odds stacked as much as possible in our favour. The authorities do their best by making, or trying to make, us anxious.

These two books attempt to describe what happens when the "anxiety promoting" machinery takes on a life of its own. But their styles and standpoints are very different. In *Culture of Fear*, Frank Furedi's thesis is that anxiety has become the defining spirit of the age. The *Zeitgeist* fastens itself to any natural disaster, accident, technological development, research finding or crime. No matter how slight a risk is involved or how few people are affected, it turns them into causes. This brings them into the realm of public policy and, worse, to the attention of do-gooders and bureaucratic busybodies.

The book's theme ambitiously suggests that the traditional moral forces in society are being replaced by ethical codes based on, or masquerading as, safety. And finally, a reluctance to take risks leads to the blunting of ambition. A fear of falling kills the desire to climb. It is an interesting although overstated idea, and is rhetorical where it should be analytical.

For instance, Furedi seems angered by the fact that small events use up more of people's emotional energy than large ones that are to him at least more worthy. A single teenage death from the drug Ecstasy matters more to us than the 100,000 deaths in the United Kingdom each year from tobacco-related illnesses, or an outbreak of Ebola virus counts more than pandemic diarrhoea.

Scientists traditionally find this incomprehensible and call for the public to be better educated about the statistics of risk. Playwrights and novelists, of course, understand it perfectly well as the essence of dramatic art. Certainly we in Britain prefer theatre to science. Look at our institutions, parliament, the monarchy, courts of law: all pure theatre. How else can you account for the BSE/CJD phenomenon? A disease affecting a handful of people has led to the decimation of a major industry and international

squabbling, based on evidence that is comprehensible to virtually no one.

All this begs the question of whether the public are really consumed with anxiety about the state of the world. Or are we merely entertained by the passing show, experiencing emotion briefly and superficially? Public concern is often a creation of the media. Good news is no news. Are we really worried about issues unless they have a direct impact on us? Jane Austen said it all about the Battle of Waterloo: "How dreadful that so many poor fellows have been killed and what a blessing one cares for none of them."

In this respect it has been said that the success of one British newspaper can be ascribed to its ability to relate any story anywhere in the world to its effect on the price of your house. This is Cassandra Chrones Moore's tactic in *Haunted Housing*, in which she chooses a series of scare stories connected with health — asbestos, radon, lead and electromagnetic fields — which make the buying and selling of houses more difficult and sometimes impossible. She uses this as a device to explore the interplay between the science, politics and economics of these public health issues and as a stick to beat the US regulatory agencies, such as the Environmental Protection Agency, for their failure to deploy rational evidence-based policies.

Haunted Housing is both scholarly — well researched and argued — and well written. One of the insights it provides is an answer to the question of how scientists know how great is the health risk in our homes. The author puts forward a convincing case that they do not, exposing the difficulties in extrapolating from one set of circumstances to another, and shows the folly of using such extrapolations as any more than the most tentative basis for public policy.

An underlying theme in both books is that science is always telling us more than we want to know, and less than we need to know to take effective action. Richard Feynman's remark that science does not come with instructions about how to use it has never been more apt. Science has lit the fuse for the information explosion, which often prevents rational argument because it becomes impossible, even for experts, to be entirely certain of their subject.

One of Furedi's lines of argument is presumably that we have given up the certainty of faith for the uncertainty of science. As a counter, Moore points out the folly of faith in scientific findings that do not warrant it. Governments and their agencies are in the same psychological bind as parents over-anxious to protect their children from the pitfalls of life rather than letting them find out for themselves. The only thing worse politically than doing something is doing nothing. □

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Simulation of modern and glacial climates with a coupled global model of intermediate complexity

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A global coupled ocean–atmosphere model of intermediate complexity is used to simulate the equilibrium climate of both today and the Last Glacial Maximum, around 21,000 years ago. The model successfully predicts the atmospheric and oceanic circulations, temperature distribution, hydrological cycle and sea-ice cover for both periods without using ‘flux adjustments’. Changes in oceanic circulation, particularly in the Atlantic Ocean, play an important role in glacial cooling.

Simulation of the icy climate of the Last Glacial Maximum (LGM) is one of the main challenges facing climate modellers. Until now, ocean models and atmosphere models have been used separately to reproduce aspects of glacial climate. For the atmospheric model simulations the sea surface temperature (SST) needs to be specified in some way, and two approaches have been used for this. The first is to use data-based reconstructions of glacial sea surface temperatures (for example, the CLIMAP reconstruction¹) as a boundary condition; eight simulations of this type are at present being evaluated in the palaeoclimate model intercomparison project (PMIP)². These experiments are diagnostic in the sense that the surface temperature of two-thirds of the planet is prescribed and no closed heat budget exists (a net global heat flux from ocean to atmosphere typically arises, shown, for example, as 4×10^{14} W in ref. 3).

The second approach is to compute SST by assuming a slab ocean of 50 m thickness with fixed (present-day or similar) heat transport; six of the PMIP simulations fall in this category, as well as the one recently published by Webb *et al.*³. However, there is no basis for assuming near-modern ocean heat transport values in a radically different climate, except as a sensitivity experiment. Ocean model studies^{4–6} and sediment core data^{7–9}, as well as our coupled simulation, suggest that ocean circulation during the LGM was different from today's, particularly the Atlantic ‘conveyor belt’, the main transporter of heat. Even if the circulation remained the same, changed temperatures would alter the heat transport.

Ocean-only model simulations suffer a similar limitation to the atmosphere-only models: they have to specify glacial surface conditions. For a credible simulation of the glacial (or any other) climate state, which predicts rather than assumes the surface temperature distribution, only coupled ocean–atmosphere models can be used. Coupled general circulation models (GCMs) are routinely used to simulate present climate and to study climate variability and possible anthropogenic changes. But most of these models still use *ad hoc* flux adjustments at the air–sea interface, which makes questionable the application of these models to climate states far from the present. Furthermore, the long (diffusive) adjustment timescale of the deep ocean requires an equilibration time of several thousand years. It is still impractical today to integrate a coupled circulation model for that long, because of the prohibitive computational cost arising from the short time-steps needed to resolve weather in the atmosphere.

We have sought a way out of this dilemma by developing an efficient climate model of intermediate complexity, which does not resolve individual synoptic weather systems but parametrizes their effects and contains the most important climate processes and

feedbacks. With this model we have conducted equilibrium simulations of both modern and glacial climate; the latter was forced by a change in solar forcing, a reduced atmospheric CO₂ concentration and prescribed continental ice sheets corresponding to 21,000 yr before present. The main characteristics of the model's glacial climate, described in detail in the following sections, are a global cooling of 6.2 °C, a cooling of the tropics of 3.8 °C (4.6 °C over land), an increase in Northern Hemisphere westerlies and trade winds, a southward shift of North Atlantic Deep Water (NADW) formation, a penetration of Antarctic Bottom Water (AABW) into the northern North Atlantic, and a substantially reduced oceanic heat transport into the high latitudes of the North Atlantic with a corresponding southward movement of the sea-ice margin to between 50° and 60° N.

Model and coupling procedure

The CLIMBER-2 model used in this study¹⁰ is a low-resolution coupled climate–biosphere model and was developed at the Potsdam Institute for Climate Impact Research (PIK) for long-term simulations. The biosphere module was not used in this study; instead, the fraction of vegetation was prescribed based on empirical data for modern conditions. Biospheric feedback will be considered in a future study.

The atmosphere module is a substantially improved version of the dynamical–statistical atmosphere model of Petoukhov and Ganopolski¹¹, with 10° latitudinal and 51° longitudinal resolution. Each grid cell can have arbitrary land and ocean fractions for which calculations are performed separately. The model explicitly resolves the large-scale circulation patterns: subtropical jet streams, Hadley, Ferrel and polar cells, monsoon and Walker circulations, tropospheric quasi-stationary planetary waves, and centres of action such as the Siberian high-pressure area and the Aleutian low-pressure area. It does not resolve individual synoptic weather systems but rather predicts their statistical characteristics, including the fluxes of heat, moisture and momentum associated with ensembles of synoptic systems. The vertical structure includes a planetary boundary layer, a free troposphere (including cumulus and stratiform clouds) and a stratosphere. Radiative fluxes are computed on 16 vertical levels. In short, the model works like most GCMs except that synoptic-scale activity is parametrized, which allows much longer time steps and coarse resolution.

The ocean module is a zonally averaged model with three separate basins (Atlantic, Indian and Pacific oceans) similar to the one used by Stocker *et al.*¹², with parametrizations of the vorticity balance and of Ekman transport. It includes a thermodynamic sea-ice model

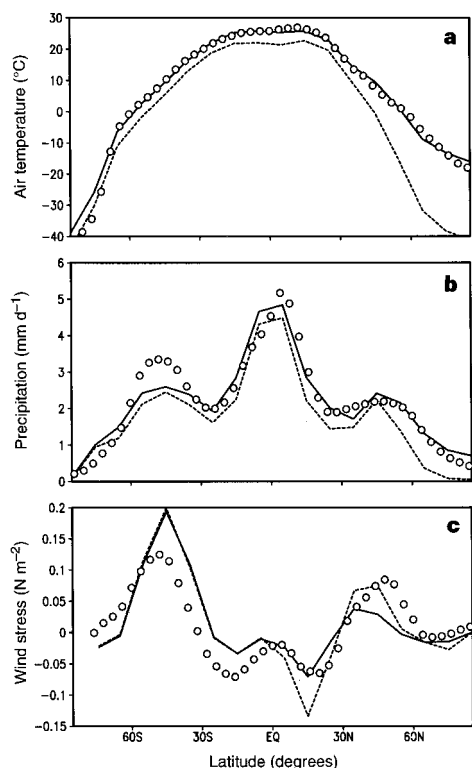


Figure 1 Zonally averaged annual-mean characteristics: **a**, air temperature; **b**, precipitation; **c**, wind stress over the oceans. The solid line shows results of the coupled model for the present climate, and the dashed line for the glacial climate. The dots indicate observed modern values^{45,46}. Model output is averaged over the last 100 years of the 5,000-year simulation. We note the decrease in temperature and precipitation, and the increase in wind stress, in the glacial climate.

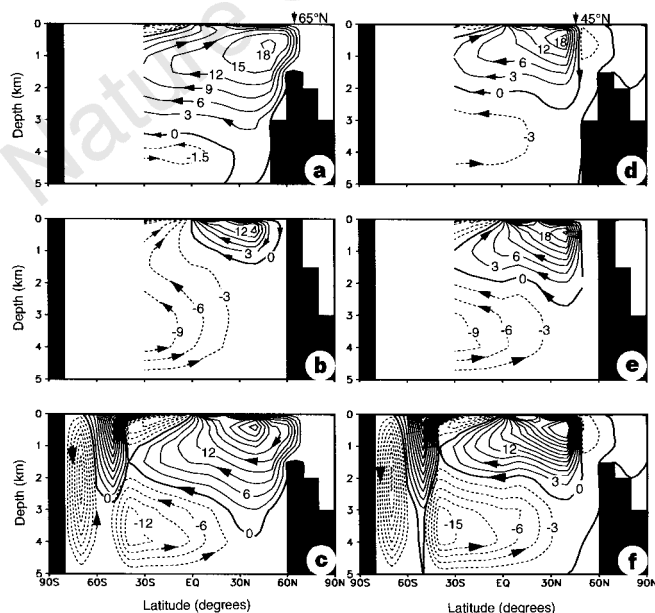


Figure 2 Meridional transport stream function for the Atlantic (top), Indo-Pacific (middle) and global ocean (bottom). The left-hand column (**a–c**) shows the modern circulation, and the right-hand column (**d–f**) shows the glacial circulation as simulated by the coupled model. Contour values are in Sv (1 sverdrup = $10^6 \text{ m}^3 \text{ s}^{-1}$).

which predicts the sea-ice fraction and thickness for each grid cell, with simple treatment of advection and diffusion of sea ice.

Ocean and atmosphere interact via surface fluxes of heat, fresh water and momentum. Freshwater fluxes into the ocean include continental runoff, routed by assigning an ocean grid cell to each land cell. Glaciers are assumed to be in equilibrium, and glacier runoff is computed as the difference between annual accumulation and ablation; it is added to the mass of sea ice in corresponding ocean areas.

The relative simplicity of the model allows coupled long-term simulations to be performed, for example the calculation of a climate equilibrium state that is not close to the initial conditions. The price to pay for the high computational speed is that only the large-scale, time-averaged properties of climate are captured. Unlike GCMs, our model cannot simulate weather, many natural variability modes (such as El Niño/Southern Oscillation), or regional details. The ocean module resolves only the vertical circulation (Ekman cells and thermohaline flow), whereas zonal gradients within each ocean are ignored and the heat and salt transports of the horizontal circulation are parametrized as simple diffusion terms.

Ocean and atmosphere modules were validated separately for the present climate against observed data, using traditional surface boundary conditions (prescribed SST and sea-ice fraction for the atmosphere module, relaxation towards observed SST and salinity for the ocean module). Both model components reproduce reasonably well the chief characteristics of the present climate state (for example, temperature, precipitation, cloudiness, energy fluxes, atmospheric and thermohaline ocean circulation), within the range shown by GCMs. In addition, systematic comparisons showed that the ocean component has a very similar response to changes in freshwater forcing as an ocean GCM¹³. An important diagnostic used in tuning the atmosphere module is the implied oceanic heat and freshwater transport (computed as an integral of air–sea fluxes), which needs to be well represented in order to allow successful coupling¹⁴.

The models were then coupled without any flux adjustments and the equilibrium climate was computed by integrating the coupled system synchronously for 5,000 model years with a fixed atmospheric CO_2 concentration of 280 p.p.m. corresponding to modern (pre-industrial) conditions. In the glacial experiment, the annual cycle of insolation was changed, the atmospheric CO_2 concentration was reduced to 200 p.p.m., continental ice sheets¹⁵ were prescribed (and consequently the sea level was lowered by 105 m and global salinity increased by 1.0 practical salinity units, p.s.u.) to reflect conditions 21,000 yr before the present (these are the standard LGM boundary conditions defined by the PMIP² project). Again, the coupled model was integrated for 5,000 model years to reach climate equilibrium.

Modern climate

After coupling, the model settled into a climate state close to that of the uncoupled component models. Some zonally averaged, annual-mean characteristics of the coupled climate are shown in Fig. 1. Seasonal variations of quantities such as temperature, precipitation, evaporation and ice cover are not shown here, but are also in good agreement with observations, as is the contrast between land and ocean areas.

The Atlantic Ocean (Fig. 2a) in the coupled simulation forms $20 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ (20 sverdrups (Sv)) of NADW, 10 Sv of this NADW flow into the circumpolar ocean at 30°S between 1.5 and 3.5 km depth. Between 4 and 5 km depth, 2 Sv of AABW enter the Atlantic from the south. These numbers are within the range of present oceanographic estimates^{16,17}. Flow in the Indian and Pacific oceans (Fig. 2b) is dominated by inflow from the south below 3 km depth, upwelling and southward return flow between 1.5 and 3 km depth (consistent with tracer data¹⁸), and northward inflow of inter-

mediate water across 30°S above 1.5 km depth. The global stream function (Fig. 2c) in addition shows the overturning cells of the Southern Ocean¹⁹: the Deacon cell (30 Sv) and the Antarctic cell (19 Sv).

The vertical salinity structure of the Atlantic (Fig. 3a) is crucial for the freshwater transport and stability of the 'conveyor belt'²⁰; it shows the characteristic fresh tongue of Antarctic Intermediate Water at around 1 km depth, in agreement with observations²¹. Oceanic heat transports are shown in Fig. 4a–c. The Atlantic heat transport is northward throughout, peaking at 1.2 PW at 20°N in agreement with oceanographic measurements²². The combined Pacific and Indian Ocean heat transport is poleward in both hemispheres, as observed²³.

Climate equilibria for present-day forcing

For present-day forcing conditions, our coupled model has the same two climate equilibrium states which were previously found in the

coupled GCM of Manabe and Stouffer²⁴, namely with NADW formation (Fig. 2a) and without (not shown). Which of these distinct states is reached depends on the initial conditions. The state without NADW formation shows a region of cooling (relative to the present climate) centred on the northern North Atlantic with a maximum of 8 °C (annual mean), with a very similar pattern but slightly weaker than that of ref. 24 (10 °C). This cooling is much stronger in winter (up to 20 °C in February).

These two different climate states provide an important sensitivity test for our atmosphere module when compared with GCM results. Our model obtains not only the same kind of temperature changes, but also very similar changes in precipitation, sea-level pressure and atmospheric circulations as the Manabe and Stouffer GCM. As an example we show the atmospheric meridional mass transport and its changes in Fig. 5. Further sensitivity studies (for example, global warming scenarios) have been performed which likewise show good agreement of our model with GCM simulations;

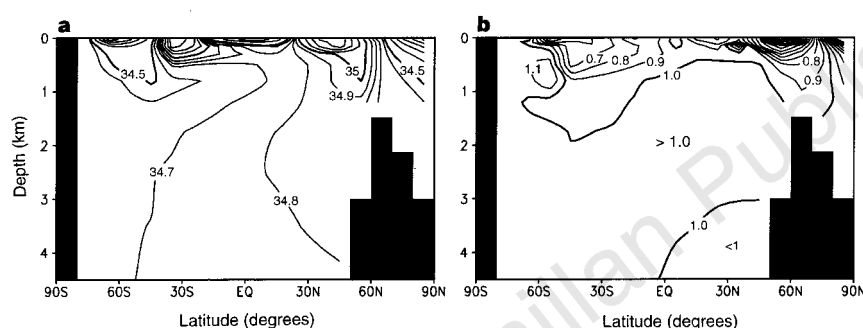


Figure 3 Zonal mean salinity in the Atlantic for the modern climate (a) and the difference between glacial and modern climates (b). The tongue of fresh Antarctic Intermediate Water spreading north at 1 km depth is shown in a. We note that global mean salinity in b was increased by 1.0 p.s.u. owing to fresh water being locked up in ice sheets; a salinity increase of <1 p.s.u. thus corresponds to a relative salinity decrease in its effects on circulation.

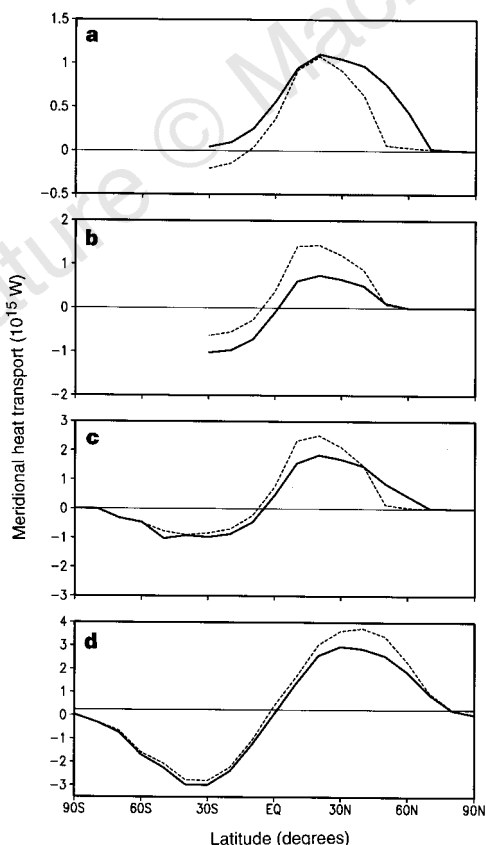


Figure 4 Meridional heat transport curves for the Atlantic (a), the Indo-Pacific (b), the global ocean (c) and the atmosphere (d). Solid lines show the simulated modern climate, dashed lines the glacial climate.

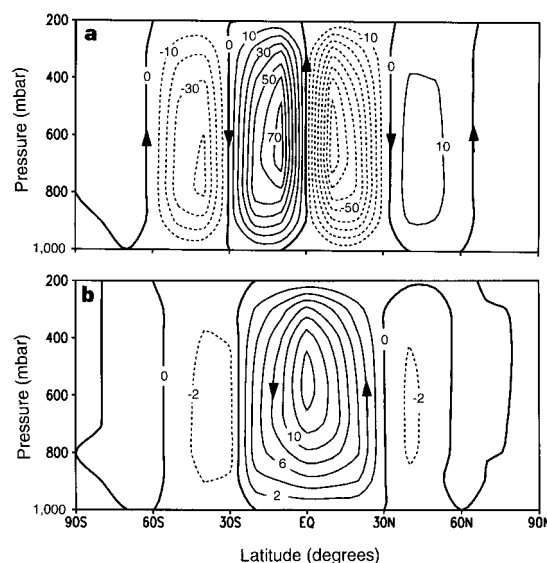


Figure 5 Annual-mean atmospheric meridional mass transport in units of 10^9 kg s^{-1} . a, The present climate; b, the difference of a climate with and without NADW formation. This difference represents a strengthening of the Northern Hemisphere Hadley circulation and a southward shift of the intertropical convergence zone (ITCZ) when NADW formation is turned off. Compare Fig. 26 of ref. 24.

these will be reported in detail elsewhere. The model's climate sensitivity to a doubling of atmospheric CO₂ content is 3.0 °C, in the middle of the range given by the IPCC²⁵.

Glacial climate

When the LGM boundary conditions are imposed, a glacial climate is simulated that is on average 6.2 °C colder (surface air temperature) than the modern (pre-industrial) climate. The spatial and seasonal distribution of the cooling is shown in Fig. 6. The strongest cooling (up to 30 °C) is over the North Atlantic and Europe in winter; in summer there are two slightly weaker maxima over the ice sheets of the northern continents. Zonal mean cooling (Fig. 7a) is up to 25 °C north of 60° N and declines towards the south, reaching just under 5 °C at the Equator and 3–5 °C in most of the Southern Hemisphere.

The tropical cooling in our coupled run is partly due to increased oceanic heat transport out of the tropics (Fig. 4a–c): the oceans transport an additional 0.7 PW northwards across 20° N in glacial conditions, whereas southward transport across 20° S is reduced slightly (Fig. 4c). The increased northward ocean heat transport out of the tropics is partly due to the enhanced meridional temperature gradient and partly due to enhanced Ekman circulation as a consequence of the stronger Northern Hemisphere trade winds. An experiment without these ocean heat transport changes (with slab ocean, discussed below) shows much less tropical cooling (Fig. 7). Further tropical cooling is due to atmospheric changes, mainly the reduction of atmospheric water vapour and CO₂ concentration.

and increased atmospheric heat transport towards northern middle latitudes (0.3 PW across 20° N, Fig. 4d).

Our model predicts substantial changes in the hydrological cycle (Fig. 6c, d). As in all models, precipitation simulations are less reliable than temperature predictions and must be interpreted with caution. Nevertheless, several significant features stand out which are also reflected in proxy data: a drying of the tropics²⁶ and northern high latitudes²⁷, a strongly reduced Asian summer monsoon²⁸, and increased winter precipitation in some (sub)tropical regions²⁶. Global mean precipitation is reduced by 16% from 2.65 to 2.23 mm d⁻¹.

The main changes in atmospheric circulation are a strengthening of the Northern Hemisphere westerlies²⁷ and trade winds²⁹ (Fig. 1c) and a southward shift in the storm track over the Atlantic (visible in the precipitation change pattern in Fig. 6c) in response to the sea-ice advance.

The simulated ocean circulation changes in the Atlantic (Fig. 2d)—southward shift of convection sites, shallower outflow of NADW, northward penetration of AABW—agree with recent reconstructions of glacial ocean circulation³⁰. We note that the overall rate of NADW formation, and its outflow into the Southern Ocean, is only slightly reduced, consistent with radiochemical data³¹. The shallower NADW flow is caused by a relative salinity and density reduction in NADW compared with AABW (Fig. 3b); the flow rate does not change much because relative salinity in the South Atlantic decreases as much as in the north, and the flow depends on the north–south density gradient²⁰. In the Pacific

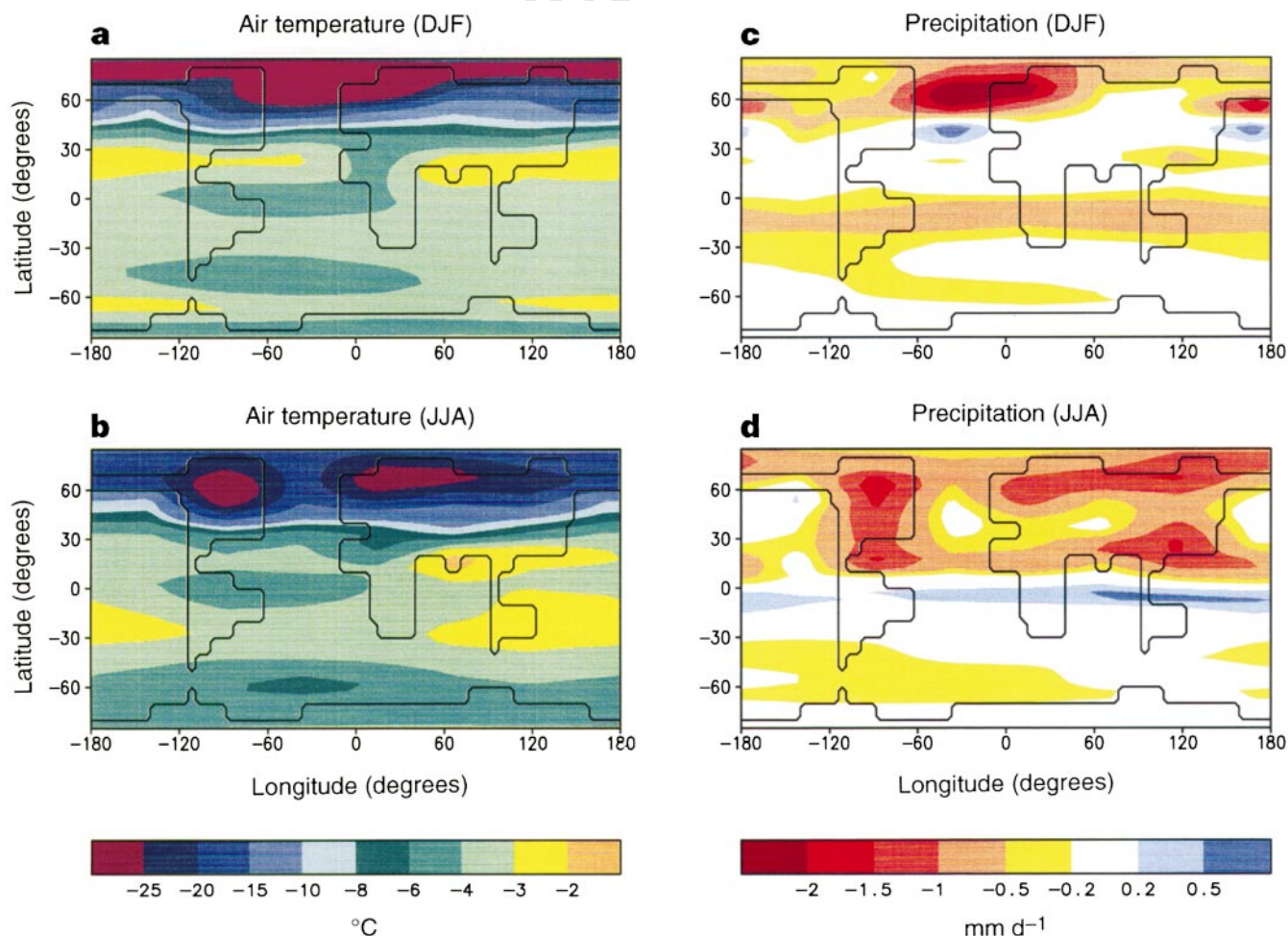


Figure 6 Temperature (a, b) and precipitation (c, d) changes in the glacial simulation relative to the modern climate simulation (LGM minus modern). Top

panels show the northern winter months (December, January, February; DJF), bottom panels show the summer months (June, July, August; JJA).

(Fig. 2e) there is little change in abyssal circulation, but the formation of intermediate water in the North Pacific is increased to the extent that Atlantic and Pacific oceans have a similar thermohaline circulation in glacial conditions³², quite unlike today. Formation of AABW (Fig. 2f) increases from 19 to 22 Sv.

For comparison, a simulation with a 50-m slab ocean and fixed (modern) ocean heat transport was performed. This represents a case where the ocean circulation plays no role in glacial cooling: slab ocean temperatures are determined strictly locally and do not depend on temperature changes elsewhere in the ocean; climate changes can be communicated only through the atmosphere. Whereas the present climate differs little between slab ocean and coupled simulation, global cooling for the LGM in the slab-ocean experiment was only 4.7°C. This shows that changes in the ocean circulation enhance global cooling by 30% in our model (in the Northern Hemisphere the cooling is even enhanced by 50%). This may seem surprising at first, given that in equilibrium the ocean cannot be a global heat sink but merely transports heat from one place to another. However, the ocean circulation can influence the global heat budget, for example through the albedo of sea ice. In the modern climate, the northern Atlantic is kept ice-free up to high latitudes (~75°N in our model, in good agreement with satellite data) owing to the northward heat transport of the thermohaline circulation. In our glacial simulation, the sites of convection and formation of NADW shifted to south of 50°N (Fig. 2d) and so did the heat release by the ocean (Fig. 4a). The sea-ice margin moved southwards to between 50° and 60°N in winter, consistent with the

CLIMAP¹ reconstruction. This increased the albedo and enhanced the high-latitude cooling by up to 10°C relative to the slab-ocean case (zonal and annual mean, Fig. 7a).

It is important to note that the cooling here does not result from an overall reduction in NADW formation, but merely from a southward shift of convection sites (as discussed by Rahmstorf³³ for a three-dimensional ocean model). The extent of sea ice and the sites of oceanic convection thus seem to be crucial regulators of climate in the northern middle and high latitudes, and even affect temperatures globally. The position of the ice margin is partly determined by oceanic heat transport; on the other hand the ice margin limits how far northward the ocean can transport heat. This interplay between ocean circulation and sea ice provides an important feedback which is linked to the ice–albedo feedback. It is only crudely modelled in our model, and regional details may become important. However, we speculate that the situation in many three-dimensional circulation models is also problematic: owing to difficulties with the representation of NADW overflow over the Greenland–Iceland–Scotland sill³⁴, these models tend to form NADW south of the sill far from the sea-ice margin, so that ice margin and thermohaline circulation become effectively decoupled. This may partly explain why even large reductions in NADW formation in global-warming scenarios (see, for example, refs 35 and 36) seem to have surprisingly little effect on climate³⁷.

Our slab-ocean experiment is comparable to the equivalent PMIP² simulations with atmospheric GCMs, and we obtain a cooling within the range simulated by the PMIP models. Much larger cooling (8.1°C globally) was simulated only by the Goddard Institute for Space Studies (GISS) model³, which seems to have a higher climate sensitivity than other models. The larger global cooling in the GISS model is due to a much colder Southern Hemisphere with massive sea-ice advance into middle latitudes. The largest zonal mean SST reduction in that model (8°C) is found at 43°S. In our experiments, glacial cooling is much stronger in the Northern than in the Southern Hemisphere, and the meridional cooling pattern of the coupled model agrees well with the CLIMAP reconstruction except for the colder tropics (Fig. 7b).

Comparison with palaeoclimate data

Our simulation of the glacial climate is, to our knowledge, the first in which atmospheric and oceanic circulation are free to evolve together and find their own equilibrium climate. Changes in ocean circulation thereby lead to enhanced glacial cooling, notably in the North Atlantic region but also in the tropics. But how realistic are these results?

In the tropical belt, the CLIMAP reconstruction gives a sea surface cooling of only 1–2°C, with some areas even warmer than at present. This is hard to reconcile with land data^{38,39} showing cooling of ~5°C. More recent marine data provide increasing evidence for a stronger cooling of the tropical oceans^{40,41}. In our coupled model, the tropics cool by an average 4.6°C over land, consistent with the data. The cooling in SST between 20°S and 20°N is 3.3°C in the Atlantic, 2.4°C in the Pacific and 1.3°C in the Indian Ocean. These values fall between the cold coral data^{40,41} and the relatively warm CLIMAP data. The fact that cooling is largest in the tropical Atlantic is in accord with palaeotemperature reconstructions⁴².

In the North Atlantic region and over Europe the differences between the coupled simulation and the one with slab ocean are largest, as oceanic heat transport towards the northern North Atlantic is dramatically reduced in the coupled model. Again, the palaeoclimate data show no consensus in this region, with more recent data⁴³ indicating colder conditions than previously thought (see, for example, ref. 44). Peyron *et al.*⁴³ distinguish a northwestern zone, north of the Pyrenees–Alps line, with an annual cooling of $12 \pm 3^\circ\text{C}$ and a maximum winter cooling of $30 \pm 10^\circ\text{C}$, and a Mediterranean zone with an annual cooling of $10 \pm 5^\circ\text{C}$, and

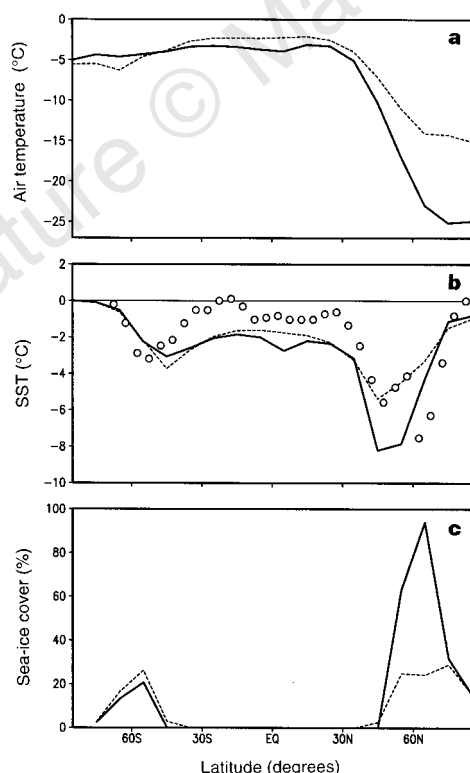


Figure 7 Glacial changes in three characteristics simulated with the coupled model (solid line) and the slab ocean (dashed line). **a**, Zonal mean surface air temperature; **b**, SST; **c**, percentage of sea-ice cover. The dots in **b** show the CLIMAP¹ reconstruction, which is much warmer in low latitudes than our model.

15 ± 5 °C cooling in the coldest month. It is difficult to compare these regional values with our low-resolution model as there is a strong gradient over Europe. Nevertheless, for northern Europe we get values close to those quoted above (Fig. 6), including the enhanced winter cooling and the pronounced drying found in the climate reconstruction from pollen data⁴³. For the Mediterranean zone (~40° N) the annual cooling is 8 °C in our model and thus within the error margin of the data; in agreement with the pollen data, we get only a moderate drying in this region.

Overall, our coupled simulation supports a relatively cold glacial climate as is emerging from the most recent palaeoclimate reconstructions. Comparison with our slab-ocean experiment shows that this is due to changes in the ocean circulation in the Atlantic, and not to a high climate sensitivity of the atmosphere model (as reference point, the equilibrium sensitivity of our model to a doubling of atmospheric CO₂ is 3.0 °C globally and 2.4 °C for the tropics).

Implications

The successful simulation of both modern and glacial climates with the same coupled model, in which the component models have only been tuned separately and for modern conditions and where no flux adjustments were used, demonstrates the ability of climate models to predict radically different climate states. This is an important precondition for a greater credibility of global warming computations.

Although our results are highly encouraging, they can only be a first step. More simulations with increasingly detailed models, as well as improved palaeoclimate data, will be required to arrive at a robust quantitative understanding of glacial climate. We also point out that in our simulation the continental ice sheets and atmospheric CO₂ concentration were prescribed. A complete climate-system model should be able to predict the growth of ice sheets and the carbon cycle. Ultimately, the challenge is thus to produce a simulation of glacial cycles driven only by the Milankovic cycles in solar forcing. □

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Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction

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The Spemann organizer in amphibian embryos is a tissue with potent head-inducing activity, the molecular nature of which is unresolved. Here we describe *dickkopf-1* (*dkk-1*), which encodes Dkk-1, a secreted inducer of Spemann's organizer in *Xenopus* and a member of a new protein family. Injections of mRNA and antibody indicate that *dkk-1* is sufficient and necessary to cause head induction. *dkk-1* is a potent antagonist of Wnt signalling, suggesting that *dkk* genes encode a family of secreted Wnt inhibitors.

Spemann and Mangold established that the organizer in amphibian embryos can be subdivided into head- and trunk-inducing tissues^{1,2}. The molecular mechanism of trunk-organizer formation involves several factors secreted from the blastopore lip that act by repressing signalling by bone morphogenetic proteins (BMPs), which antagonizes the organizer³. Less is known about the molecular basis of head induction. The secreted factor Cerberus can induce ectopic heads without trunks⁴, and mice mutant for the genes *lim-1* or *otx2* do not form heads although they form a relatively normal trunk⁵. These results support the idea that head formation can be uncoupled from trunk formation in vertebrates, and argues for the presence of distinct inducers.

We recently suggested a two-inhibitor model, in which head-organizer activity requires the repression of signalling by Wnt as well as by BMP⁶. Members of the *Wnt* gene family encode secreted glycoproteins that are required for a variety of developmental processes, ranging from cell-lineage decisions in *Caenorhabditis elegans* to control of differentiation of the central nervous system in higher vertebrates. In the adult vertebrate, *Wnt* genes are implicated in tissue homeostasis and oncogenesis^{7,8}. In *Xenopus* embryogenesis, Wnt signalling is involved in dorsoventral axial patterning at two stages, before and after the midblastula transition (MBT)⁹, which we refer to as early and late Wnt signalling, respectively. The early Wnt signalling pathway is thought to mediate the dorsalizing function of the Nieuwkoop centre, whereas late Wnt signalling is thought to be involved in ventrolateral mesoderm patterning. Consistent with our two-inhibitor model for head formation, the organizer secretes at least one Wnt-antagonist, Frzb, which blocks muscle formation and anteriorizes embryos^{10,11}.

Here we describe *dickkopf-1* (*dkk-1*; German, big head, stubborn), a member of a new gene family that encodes secreted proteins. *dkk-1* is expressed in the head organizer, and mRNA co-injection with BMP inhibitors leads to the induction of complete head structures in *Xenopus* embryos. In contrast, injection of anti-Dkk-1 antibodies leads to microcephaly. Dkk-1 is thus a growth-factor antagonist of the Spemann organizer and is required for head formation. Dkk-1 is also a potent Wnt antagonist. The possibility that *dkk* genes encode a new family of Wnt inhibitors has important implications for Wnt-regulated embryonic patterning, cell differentiation, and tissue homeostasis.

Expression cloning of *dickkopf-1*

A cDNA library¹² was screened by microinjecting pools of synthetic mRNA with or without mRNA encoding the dominant-negative Bmp-2/4 receptor (tBR)¹³ into two opposite blastomeres of four-

cell-stage embryos. tBR injection alone induces incomplete secondary embryonic axes that lack a head. Pools of mRNA were selected that were inactive when injected alone, but induced complete secondary axes when injected with tBR. Sib selection led to an individual cDNA, *dkk-1*, which shows no homology to genes with known function. It encodes a protein of 259 amino acids that contains a signal sequence and two cysteine-rich domains (Fig. 1a). Mouse cDNA library screening and expressed sequence tag (EST)-sequence database searches revealed that *Xenopus dkk-1* is member of a new multigene family with at least three members, *dkk-1*, -2 and -3, present in different species (Fig. 1b). The cysteine-rich domains are highly conserved among members of the family, displaying between 80% and 90% amino-acid sequence similarity. *dkk-1* expression is inhibited by ultraviolet irradiation and induced by LiCl treatment, like other typical organizer genes (*cerberus*⁴ and *chordin*¹⁴) (Fig. 1c).

Dkk-1 can be immunoprecipitated as a protein of relative molecular mass 40,000 (M_r 40K) from the medium of oocytes injected with FLAG-tagged *dkk-1* mRNA, indicating that Dkk-1 is indeed secreted (Fig. 1d). The major band in the cell extract migrates as a protein of 35K, suggesting that Dkk-1 is post-translationally modified before secretion. Dkk-1 appears to be a monomer, as under non-reducing conditions the mobility of the proteins is lower than the reduced form.

Antibodies were raised against two synthetic peptides (see Fig. 1a). In extracts from embryos microinjected with *dkk-1* mRNA, the polyclonal antibody anti-15 recognizes both intracellular and extracellular Dkk-1, whereas the antibody anti-14 predominantly recognizes the smaller intracellular protein (Fig. 1e). This suggests that the carboxy-terminal epitope is masked or modified upon secretion.

Expression of *dkk-1* in anterior organizer

By whole-mount *in situ* hybridization the earliest expression of *dkk-1* is seen in the early gastrula, where the gene is expressed in the Spemann organizer (Fig. 2a). In comparison, the head inducer *cerberus* is expressed in a wider dorsolateral arc (Fig. 2b). Within the organizer, expression is detected in the deep cells at stage 10 but excluding the endoderm (not shown). At late gastrula stage, *dkk-1* is expressed in three domains (Fig. 2c, d), with the most anterior expression being in the leading edge cells of the involuting cylinder, which will give rise to the liver. Strongest expression is found in a wing-shaped middle domain of endomesoderm corresponding to prospective prechordal plate. The Wnt antagonist *frzb* is strongly expressed at the same anterior level (Fig. 2e). This tissue is the most potent head inducer^{2,4}. In a posterior third domain, *dkk-1* is

expressed in two longitudinal stripes (Fig. 2c), flanking the anterior chordamesoderm, partly overlapping *chordin* expression (Fig. 2f). Neither *frzb* nor *dkk-1* are expressed in posterior chordamesoderm, unlike *chordin*. Prospective chordamesoderm induces predominantly trunk structures². These posterior *dkk-1*-expressing cells may correspond to adaxial muscle pioneer precursors¹⁵. At late-neurula stage, *dkk-1* shows strongest expression in the prechordal plate adjacent to prospective forebrain and eyes (Fig. 2g). In addition, expression occurs in a stripe corresponding to the forming somites.

Mouse *dkk-1* (*mdkk-1*) transcripts are first detected on postcoital day 6.5 (P6.5) in mesodermal cells adjacent to the embryonic/extraembryonic junction (Fig. 2h, i). These cells are derived from the primitive streak and migrate laterally and anteriorly to form the extraembryonic and head-fold mesoderm. Over the next 24 h, the number of *mdkk-1*-positive cells increases in the developing head

fold, while cells in the primitive streak do not express *mdkk-1* at this time (Fig. 2i, j). This suggests that, like its *Xenopus* homologue, *mdkk-1* marks head mesoderm cells. Between days P7 and P8, *mdkk-1* transcripts are initially detected only in head mesoderm (Fig. 2j), but on P8 weak signals are detected in posterior primitive-streak mesoderm (Fig. 2k), and on P8.5 in developing somites (not shown), corresponding to the somitic expression observed in *Xenopus*. At this stage strong expression is also observed in the foregut endoderm underlying the head fold (Fig. 2k). We conclude that *dkk-1* is expressed prominently in the tissues known to contain head organizer activity.

Table 1 *dkk-1* induces heads and inhibits early Wnt signalling

Experiment	Material injected into embryo (pg per blastomere)	Incomplete secondary axis (%)	Secondary axis with eye and cement gland (%)	No. of embryos
1.1	tBR (250)	36	0	97
1.2	<i>dkk-1</i> (12.5)	0	0	61
1.3	<i>dkk-1</i> (12.5) + tBR (250)	3	43*	96
1.4	<i>mdkk-1</i> (500)	6	0	36
1.5	<i>mdkk-1</i> (500) + tBR (250)	0	55	29
1.6	preprolactin (3,500)	0	0	50
1.7	<i>frzb</i> (100)	4	0	53
1.8	<i>frzb</i> (100) + tBR (250)	11	36	55
2.1	<i>noggin</i> (25)	31	0	42
2.2	<i>noggin</i> (25) + tBR (250)	33	0	42
2.3	<i>chordin</i> (50)	48	0	44
2.4	<i>chordin</i> (50) + tBR (250)	36	0	33
3.1	pCSdkk-1 (DNA) (25)	0	0	46
3.2	pCSnoggin (DNA) (5)	45	0	84
3.3	pCSdkk-1 (25) + <i>noggin</i> (DNA) (5)	0	57*	51
4.1	<i>Xwnt-8</i> (1.5)	0	61*	49
4.2	<i>dkk-1</i> (12.5) + <i>Xwnt-8</i> (1.5)	0	0	45
4.3	<i>dkk-1</i> (12.5) + <i>Xwnt-8</i> (12.5)	0	0	33
4.4	<i>mdkk-1</i> (500) + <i>Xwnt-8</i> (1.5)	3	0	34
4.5	<i>Xwnt-8</i> (1.5) + <i>frzb</i> (25)	0	0	35
5.1	<i>Xdsh</i> (750)	6	54*	112
5.2	<i>Xdsh</i> (750) + <i>dkk-1</i> (12.5)	6	52*	66
5.3	<i>Xdsh</i> (750) + <i>dkk-1</i> (500)	0	81†	42
6.1	<i>dn GSK3</i> (750)	3	42*	36
6.2	<i>dn GSK3</i> (750) + <i>dkk-1</i> (200)	3	56*	62
7.1	<i>plakoglobin</i> (250)	0	50*	34
7.2	<i>plakoglobin</i> (250) + <i>dkk-1</i> (12.5)	0	61*	31
7.3	<i>plakoglobin</i> (250) + <i>dkk-1</i> (50)	0	53*	28
8.1	<i>siamois</i> (12.5)	0	56*	54
8.2	<i>siamois</i> (12.5) + <i>dkk-1</i> (200)	8	43*	53

Four-cell embryos (experiments 1–3) were microinjected into two ventral blastomeres with mRNA or DNA constructs as indicated. In experiments 4–8 one ventral vegetal blastomere was injected at the eight-cell stage. Embryos were scored for the formation of incomplete or complete secondary axis. A minimum of two independent experiments was carried out for every injection; *n* is the total number of embryos analysed. Experiment 1, *dkk-1* and *frzb* synergize with the dominant-negative Bmp-receptor (tBR); 2, *noggin* and *chordin* do not synergize with tBR in head formation; 3, head induction occurs after the MBT; 4, *dkk-1* inhibits Wnt signalling; 5, *dkk-1* cannot inhibit *Xdsh*; 6, *dkk-1* cannot inhibit dominant-negative *GSK3*; 7, *dkk-1* cannot inhibit *plakoglobin*; 8, *dkk-1* cannot inhibit *siamois*.

* More than 50% of secondary heads have two separated eyes.

† Embryos had either secondary axes with eyes or showed radial cement gland.

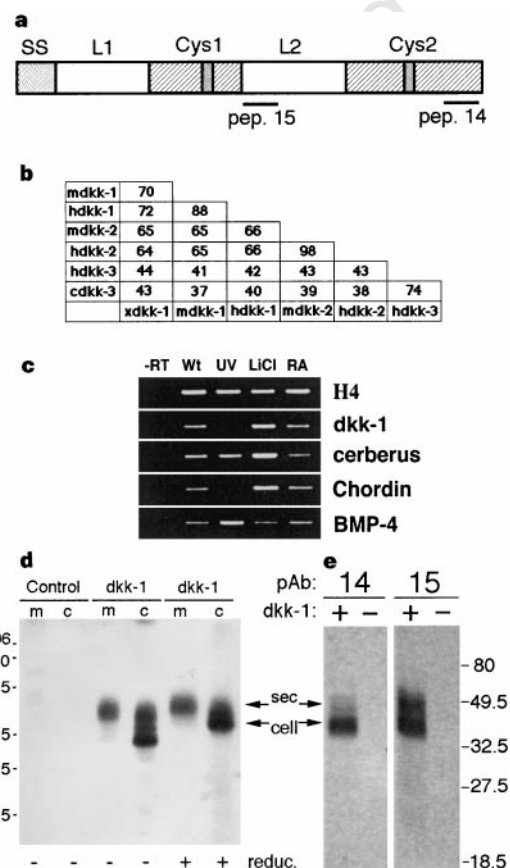


Figure 1 Dkk-1 is secreted and is a member of a new protein family. **a**, Domain structure of Dkk-1; the putative protein contains two cysteine-rich domains (Cys 1 and Cys 2), conserved among different family members. SS, signal sequence; L1 and L2, linkers 1 and 2. Positions from which peptides 14 and 15 were derived for use in immunization are marked by a line. **b**, Identities of amino-acid sequences (%) encompassing the Cys1 to Cys2 domain among Dkk family members. Consensus amino-acid sequences for the Cys domains are Cys1 (CX₁CX₂CX₃CX₄CX₅CCX₆CX₇GXC) and Cys2 (GX₁GX₂CX₃DCX₄GXCX₅CX₆PX₇GX₈CX₉RCX₁₀CX₁₁GLXC), where X_i indicates sequence stretches of variable length. **c**, *dkk-1* acts like an organizer gene. Embryos were ventralized using ultraviolet, dorsalized using LiCl or treated with 2 μM retinoic acid from stage 8 until stage 10 (RA). At early gastrula stage the expression of the indicated marker genes was analysed by RT-PCR. Histone H4 was used for normalization. Wt, wild-type embryos; -RT, minus reverse transcriptase control. **d**, Dkk-1 is secreted. Uninjected (control) or *dkk-1*-FLAG mRNA-injected (dkk-1) oocytes were incubated in ³⁵S-methionine. Proteins were immunoprecipitated with anti-FLAG antibody from cells (c) and medium (m), and analysed by SDS-PAGE under reducing and non-reducing conditions as indicated. The secreted (sec) and cellular (cell) forms are indicated. **e**, Four-cell embryos injected (+) or uninjected (–) with 2 ng *dkk-1* were analysed by western blot at stage 8. The equivalent of one embryo was loaded. The blot was probed with anti-14 (14) and anti-15 (15) antibodies raised against Dkk-1. The secreted (sec) and cellular (cell) forms of Dkk-1 are indicated; reduc., reducing conditions.

dkk-1 is a head inducer

To investigate its biological effects, *dkk-1* mRNA was microinjected radially into all blastomeres of four-cell *Xenopus* embryos. This led to anteriorized embryos with big heads, enlarged cement glands, and short trunks (Fig. 3a). Muscle differentiation was reduced and notochord formation enhanced in these embryos (data not shown). In ventral injections no secondary axes were observed with *dkk-1* (Table 1, experiment 1). However, when *dkk-1* was co-injected ventrally with tBR mRNA, embryos developed complete secondary axes with two eyes, cement glands and two beating hearts (Fig. 3b, c; Table 1, experiment 1). tBR alone, in combination with *noggin*, or in combination with *chordin* yields only incomplete secondary embryonic axes (Table 1, experiments 1 and 2). Mouse *dkk-1* is also effective in inducing complete secondary axes together with tBR (Fig. 3d), as is *frzb*, although *frzb*-induced heads are less complete, typically containing only one eye (Table 1, experiment 1).

Expression after MBT by plasmid DNA co-injection of *dkk-1* and pCSnoggin also induces complete head structures (Fig. 3e; Table 1, experiment 3). Finally, *dkk-1* mRNA injection rescued ultraviolet-ventralized embryos from a dorso-anterior index (DAI)¹⁶ of 1.3 ($n = 243$) to a DAI of 7.2 ($n = 47$; 50 pg) and 7.3 ($n = 36$; 250 pg), respectively (Fig. 3f). We conclude that *dkk-1* anteriorizes embryos

and is able to induce head structures in synergy with inhibitors of BMP signalling.

To investigate molecularly the observed phenotypes, marker gene expression was analysed. Injection of tBR mRNA dorsalizes ventral marginal zone (VMZ) mesoderm, as judged by the expression of the organizer marker *otx2* in gastrula VMZ and the muscle marker tropomyosin in tailbud stage VMZ, but *dkk-1* alone does not induce dorsal markers (Fig. 4a, b). However, *dkk-1* mRNA synergizes strongly with tBR to induce the organizer genes *gsc*¹⁷, *chordin*¹⁴ and *otx2* (refs. 18, 19) in gastrula VMZ as well as anterior neural marker in tailbud stage VMZ (*otx2*, *XAG1* (ref. 20) and *CG13* (ref. 21)) (Fig. 4a, b). Dominant-negative *xwnt-8* (dnXwnt-8) also synergizes with tBR but, unlike *dkk-1*, it can induce on its own the muscle marker tropomyosin and neural markers (Fig. 4a, b). Thus *dkk-1* is not by itself dorsalizing the VMZ, but is able to act in concert with BMP antagonists to induce organizer genes and anterior neural gene expression. Importantly, *dkk-1*, alone or in combination with tBR, is unable to induce *siamois* (Fig. 4a), a downstream target of the early Wnt pathway^{22,23} that is thought to induce the organizer. Thus, *dkk-1* appears to act downstream of *siamois* by directly conferring head-organizer activity.

To analyse the effect of expressing *dkk-1* in ectoderm, animal-cap

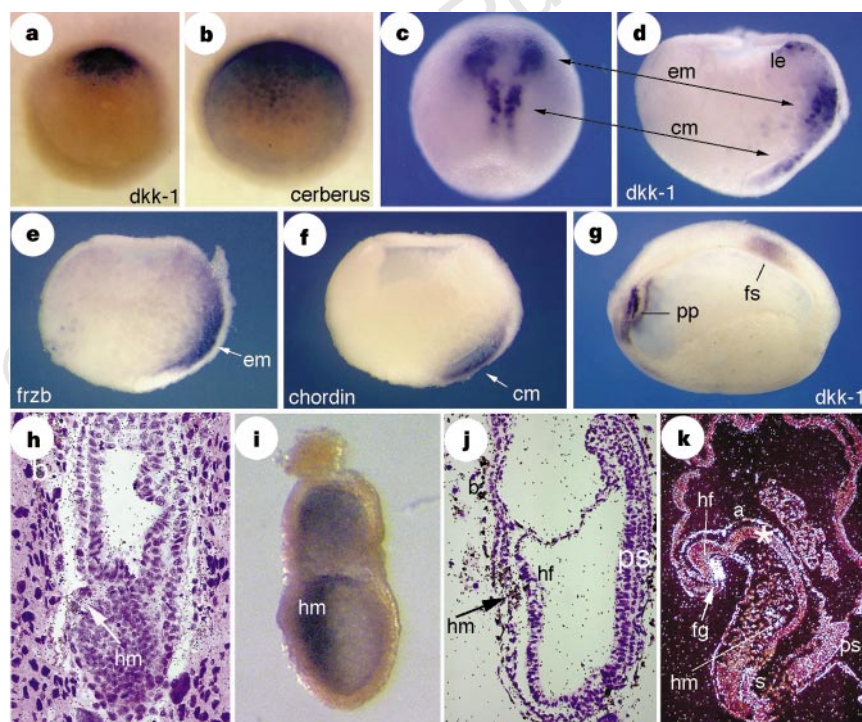


Figure 2 Expression of *dkk-1* in *Xenopus* and mouse. *In situ* hybridizations of embryo whole-mount are shown in **a–c, i** and of sections in **d–h, j, k**. *Xenopus* (**a–g**) and mouse *dkk-1* expression (**h–k**) are shown. **a**, Expression of *dkk-1* in an early gastrula in vegetal view with the dorsal side facing up. The embryo was cleared in Murrays clear. **b**, Expression of *cerberus* in an early gastrula in vegetal view with the dorsal side facing up. The embryo was cleared in Murrays clear. **c, d**, Expression of *dkk-1* in late gastrulae (stage 12). Embryos are shown with animal side up, blastopore down. Arrows point to corresponding domains in **c** and **d**. Three domains can be distinguished in dorsal view (**c**) and sagittal section (**d**, dorsal to the right). Most anteriorly the leading edge cells (le) are expressing *dkk-1* (**d**). In **c** the leading edge domain positive for *dkk-1* is out of the field of view. More posterior, endomesoderm (em) is stained in a wing-shaped pattern and most posteriorly expression is in two longitudinal stripes adjacent to the chordamesoderm (cm), possibly corresponding to adaxial cells. The expression does not reach the blastopore. **e**, *frzb* expression in same view and stage as the embryo in **d**. **f**, *chordin* expression in the same view and stage as the embryo in **d**.

g, *dkk-1* expression in late neurula (stage 16). Sagittal section with anterior to the left, dorsal side up. Expression occurs in the prechordal plate (pp) and a stripe of forming somites (fs). **h–k**, *dkk-1* expression in mouse embryo. Anterior is always to the left. In **h, j**, a dark-field image was contrast-inverted and superimposed on a bright-field image using Adobe Photoshop image program. **h**, Radioactive *in situ* hybridization showing expression in a subpopulation of prospective head mesoderm cells (hm) on day P6.5. **i, j**, Hybridization of *mdkk-1* in whole-mount (**i**) and sectioned embryo (**j**) on day P7. Transcripts are restricted to head mesoderm (hm) (arrow). Cells in the primitive streak (ps) do not express *mdkk-1*. **k**, On day P8 foregut endoderm cells (fg) adjacent to the head fold (hf) strongly express *mdkk-1*. Only a few head mesodermal (hm) cells continue to express at this stage. Two *mdkk-1*-positive domains are detected in the neuroectoderm: a small rostral domain in the diencephalon (asterisk) and a weaker ventral domain in the developing spinal cord (s). Blood cells (b) that naturally reflect light are negative for *mdkk-1* (**h, j**). Some cells in the amnion (a) also express *mdkk-1*.

assays were done. As described⁴, the head inducer *cerberus* induces pan-neural and anterior neural markers (Fig. 4d). As shown for other Wnt inhibitors^{6,24}, *dkk-1* can directly induce neural markers (*N-CAM* and *N-tubulin*) in the absence of mesodermal gene expression (*Xbra*²⁵, *actin* and α -globin²⁶) (Fig. 4b). This induction is not accompanied by histodifferentiation (data not shown), and the markers induced are of anterior (*otx2*, *XAG1* and *CG13*) not posterior character (*Hox B9/XlHbox6*). Thus *dkk-1* is able to induce a 'pre-neural' fate in animal-cap ectoderm.

dkk-1 inhibits Wnt signalling

To test directly whether *dkk-1* can antagonize early Wnt signalling, we investigated whether complete secondary-axis induction by *Xwnt-8* could be rescued by co-injected *dkk-1*. Axis induction by *Xwnt-8* is completely inhibited by *dkk-1* (Fig. 5a, b; Table 1, experiment 4). Even 10-fold higher doses of *Xwnt-8* than minimally required to induce secondary axes are insufficient to restore axis induction.

dkk-1 also inhibits late Wnt signalling. Injection of plasmid DNA pCSKA-X8 leads to expression of *Xwnt-8* after MBT. Such embryos develop with a truncated head, lacking eyes and a cement gland (93%, $n = 88$) (Fig. 5c). When pCSKA-X8 was co-injected with *dkk-1*, head formation with two eyes and a cement gland was restored (94%, $n = 54$) (Fig. 5d). Thus *dkk-1* inhibits early and late action of *Xwnt-8*.

Inhibition of Wnt signalling by *dkk-1* could occur at the extracellular level, as is the case for *frzb*^{10,11}, or intracellularly, as is the case for *axin*²⁷. Expression of the intracellular components of the Wnt pathway *Xdsh*²⁸, dominant-negative GSK-3 (refs 29, 30), the β -catenin homologue *plakoglobin*³¹ and *siamois*^{22,23} all induce complete secondary embryonic axes. To test at which level *dkk-1* interferes with Wnt signalling these mRNAs were co-injected individually with *dkk-1*. We found that *dkk-1* is unable to rescue

secondary axis formation by any of these mRNAs (Fig. 4e, f; Table 1, experiments 5–8). We conclude that *dkk-1* interferes with the transmission of the Wnt signal upstream of *Xdsh*, and thus must act extracellularly.

dkk-1 is required for head formation

To test the requirement for *dkk-1* in head formation we took an antibody-inhibition approach using polyclonal anti-peptide antibodies raised against Dkk-1. Injection into the blastocoel of pre-immune antibody ($n = 47$) or anti-14 antibody ($n = 45$), which recognizes cellular Dkk-1 (Fig. 1e), had no detectable phenotypic effect (Fig. 6a). Dramatically, microinjection of anti-15 antibody led reproducibly to microcephaly in 100% ($n = 47$) of the embryos (Fig. 6b). Typically embryos had small or no cement glands at early

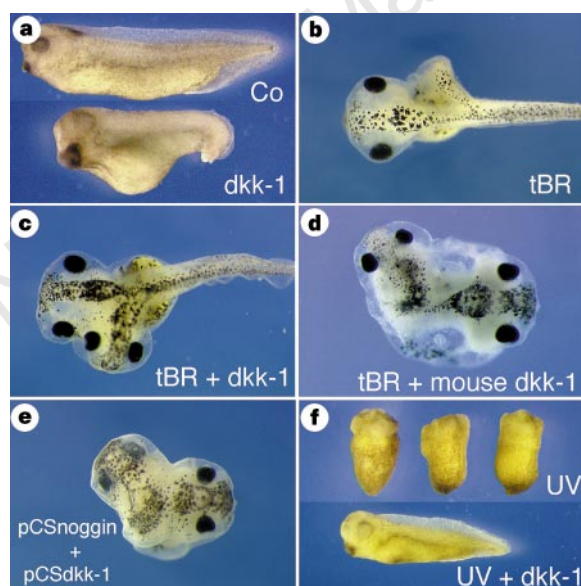


Figure 3 Head induction by *dkk-1*. Four-cell embryos were uninjected (a, control), radially injected into all blastomeres (a, *dkk-1*; f, ultraviolet treatment (UV) and *dkk-1*) or injected in two ventral blastomeres with the RNA or DNA indicated (b–e). The final doses per blastomere were 50 pg (a, f), 12.5 pg (c) *dkk-1* mRNA or 25 pg pCSdkk-1 DNA (e), 250 pg tBR mRNA (b–d), 500 pg mouse *dkk-1* mRNA (d), 5 pg pCSnogg DNA (e) as indicated.

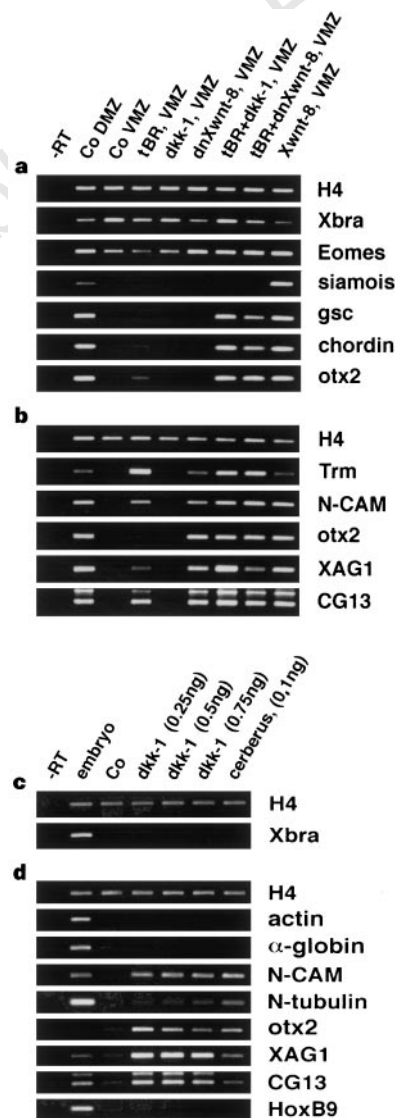


Figure 4 Molecular markers induced by *dkk-1*. a, b, VMZ assays. Four-cell embryos were microinjected into all blastomeres with 250 pg tBR, 25 pg *dkk-1*, 300 pg dnXwnt-8 or a combination as indicated. *Xwnt-8* mRNA was at 1.5 pg per blastomere. Dorsal (DMZ) or ventral marginal zone (VMZ) fragments were cut from early gastrulae and analysed at stage 10.5 (a) or at stage 32 (b) by RT-PCR. Note that *siamois* is induced by *Xwnt-8* only. c, d, Animal-cap assays. Four-cell embryos were microinjected into the animal region of all blastomeres with the doses per blastomere indicated. Animal-cap fragments were cut from late blastulae, cultivated until stage 10.5 (c) or stage 32 equivalent (d) and analysed by RT-PCR for expression of marker genes indicated. –RT, minus reverse transcription control sample. Co, control.

stages and were cyclopic at tadpole stage, but 5% of the embryos showed complete deletion of head structures (data not shown). Histological sections (Fig. 6c, d) show that head-mesoderm and cartilage formation were very reduced. In contrast to the head, the embryonic axis is unaffected by injection of anti-15 antibody. Embryos had a normal-sized trunk (Fig. 6b) and histological analysis showed that they contained apparently normal mesodermal derivatives (Fig. 6c, d, and data not shown).

Anti-15 antibody-induced microcephaly was very efficiently rescued by injection of *dkk-1* mRNA (79% normal embryos, $n = 114$) but not by preprolactin (97% microcephaly, $n = 114$), and thus is specific for *dkk-1* (Fig. 6e, f). The microcephaly phenotype was completely rescued by co-injection of 150 ng Dkk-1 antibodies with 75 ng peptide 15 (98% normal, $n = 50$), but not of peptide 14 (100% microcephalic, $n = 59$). Although only genetic loss-of-function is a rigorous test, the results strongly suggest that *dkk-1* is required for head formation.

Discussion

dkk-1 is expressed in the Spemann organizer, and our data strongly suggest that it functions as a head inducer because: it is secreted; it anteriorizes embryos and induces complete secondary axes with heads in cooperation with BMP antagonists; it is expressed at the right time in the endomesoderm to function as a head inducer; and antibody injections suggest that it is required for head formation. The *dkk-1* gene is a potent inhibitor of Wnt signalling and acts without inducing *siamois*, a downstream target of the early Wnt pathway, indicating that it confers head-organizer activity directly, downstream of *siamois*. The expression pattern and functional properties of *dkk-1* support the two-inhibitor model, according to which head induction by endomesoderm requires simultaneous inhibition of BMP and Wnt signalling⁶.

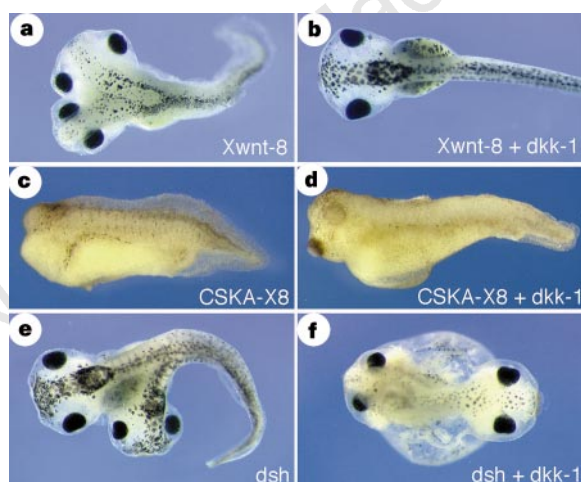


Figure 5 *dkk-1* inhibits Wnt signalling. Eight-cell embryos were injected in one ventral vegetal blastomere (a, b, e, f) or into all four blastomeres (c, d) with the RNA or DNA indicated. Embryonic phenotypes resulting from the injections are shown. a, Injection of 1.5 pg per blastomere *Xwnt-8* mRNA. b, Co-injection of 1.5 pg per blastomere *Xwnt-8* mRNA and 12.5 pg *dkk-1* mRNA. c, Injection of 37.5 pg per blastomere *CSKA-X8* DNA. d, Co-injection of 37.5 pg per blastomere *CSKA-X8* DNA and 25 pg *dkk-1* mRNA. e, Co-injection of 750 pg per blastomere *Xdsh* mRNA. f, Co-injection of 750 pg per blastomere *Xdsh* mRNA and 500 pg *dkk-1* mRNA. *dkk-1* rescues phenotypes elicited by early and late Wnt signalling but does not rescue the *Xdsh* phenotype.

Wnt antagonists affect not only mesoderm but also ectoderm directly. In animal caps, inhibition of Wnt signalling by *GSK-3* (ref. 24), *dnXwnt-8* (ref. 6), and *dkk-1* leads to induction of anterior neural markers, similar to BMP antagonists³. Because gastrula ectoderm does not express *Xwnt-8* (refs 32, 33) there are likely to be additional Wnt proteins with antineuralizing action in the ectoderm, such as that encoded by *Xwnt-8b* (ref. 34).

In *Xenopus*, *frzb* antagonizes Wnt signalling^{10,11} and induces heads when co-injected with tBR. The head inducer *cerberus* is a potent inhibitor of *Xwnt-8*-induced axis formation, suggesting that it may also interfere with Wnt signalling¹². Yet, although *cerberus*, *frzb* and *dkk-1* are implicated in head-organizer function and Wnt signalling, their properties differ markedly. Compared with *dnXwnt-8* and *frzb*, *dkk-1* seems to be more potent because induced heads contain two eyes instead of one, and *dkk-1* rescues ultraviolet-treated embryos more completely than does *frzb*^{10,11}. Furthermore, unlike *dnXwnt-8*, *dkk-1* alone does not induce anterior neural marker gene expression in VMZ. In contrast, *cerberus* induces secondary heads without additional axes, does not rescue ultraviolet-treated embryos, and inhibits primary axis formation⁴. These differences may be the result of different mechanisms of action. *Frzb* protein binds directly to *Xwnt-8* protein, but the mechanism of *cerberus* and *dkk-1* action is unclear. Both may activate signalling pathways that inhibit Wnt signalling indirectly. If they do bind Wnt proteins or frizzled-receptors³⁵ directly, they may have distinct binding specificities.

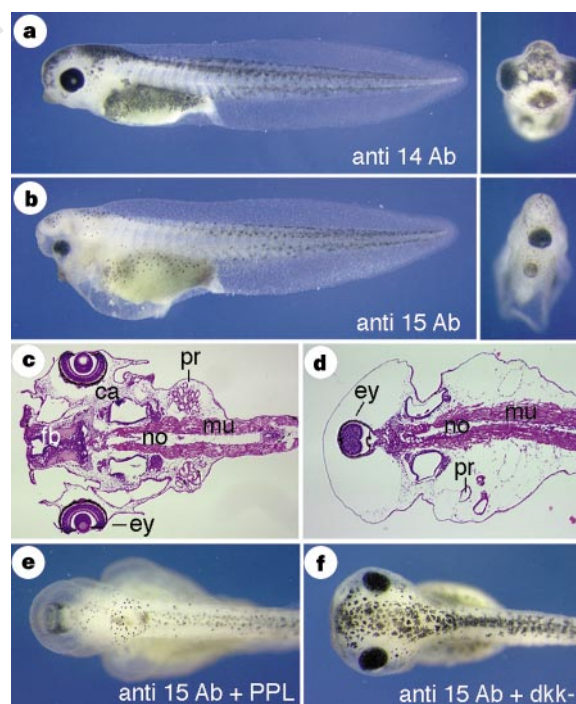


Figure 6 *dkk-1* is required for head formation. a, b, d, Stage 9 embryos were injected with 150 ng affinity-purified antibody (Ab) into the blastocoel and allowed to develop for 3 days. a, Embryos injected with anti-14 antibody show no abnormalities. An anterior view is shown on the right. b, Embryos injected with anti-15 antibody show microcephaly and cyclopia. An anterior view is shown on the right. Note that trunk and tail are unaffected. Embryos lack differentiated pigment cells. c, d, Tangential histological sections. c, Control embryo. d, Embryo injected with anti-15 antibody. Note cyclopia and under-developed head mesodermal derivatives. e, Embryo injected at the four-cell stage with 50 pg preprolactin (PPL) mRNA per blastomere in all blastomeres and at stage 9 with anti-15 antibody (210 ng). f, Embryo injected at the four-cell stage with 10 pg *dkk-1* mRNA per blastomere in all blastomeres and at stage 9 with anti-15 antibody (210 ng). Note that there is rescue of complete head structures and pigment cells. Abbreviations: ca, cartilage; ey, eye; fb, forebrain; mu, muscle; no, notochord; pr, pronephros.

However, an intracellular route of *dkk-1* action is unlikely because it blocks the Wnt pathway upstream of *Xdsh*, and thus probably by interacting with a Wnt protein or Wnt receptors³⁵.

The cooperation of BMP and Wnt signalling in patterning of the body axis is reminiscent of appendage formation in *Drosophila*, where *decapentaplegic* and *wingless* work cooperatively to organize the proximodistal axis of the leg³⁶.

It is odd, though, that the vertebrate head, which is thought to be a novel evolutionary structure³⁷, should form by default, in the absence of instructive Wnt and BMP signalling. □

Methods

Embryos and explants. *In vitro* fertilization, embryo culture, staging, microinjection and culture of explants, and treatment with LiCl and ultraviolet were performed as described³⁸. Whole-mount and radioactive *in situ* hybridizations were performed as described^{38,39}.

cDNA library screening. A plasmid cDNA library from LiCl-treated stage-13 embryos was screened¹². mRNA (5 ng) from 150 clones was microinjected in two opposite blastomeres of four-cell stage embryos with or without 250 pg tBR mRNA. Embryos were scored for secondary embryonic axes with cement glands and active pools fractionated by sib selection¹². The *Xenopus dkk-1* clone isolated is referred to as pRNdkk-1. The *dkk-1* open reading frame was subcloned into pCS2⁺ (pCsdskk-1)⁴⁰ and pSP35T-FLAG to add a C-terminal FLAG-tag (pdkk-1FLAG). In mRNA microinjections all constructs had similar effects. Plasmids were linearized and transcribed *in vitro* with the Megascript kit (Ambion). *mdkk-1* was isolated from a mouse embryonic day 9 cDNA library by the RZPD (clone MPMGc559P2481Q3) using a *Xenopus dkk-1* probe.

RT-PCR assays. Reverse transcription-polymerase chain reaction (RT-PCR) assays were carried out in the exponential phase of amplification as described³⁸. Primers used were: *Eomesodermin*⁴¹ (f, CCATCCAAACCTCCCACC; r, CTTCTCTTACATGCACCCG); *tropomyosin*⁴² (f, AAATCTCTGGAGGCC-CAGGCAA; r, GATCCAGTCTCTTCACTGATGGC); *N-tubulin*⁴³ (f, GATTCC-CAACAATGTGAAGACC; r, CAGTGTACCACTGCAAGAAGC); mouse histone 4 (*H4*) (ref. 44) (f, ATGTCTGGACGTGGCAAGGGTGG; r, CGAA-TCCGTAGAGAGTGCAGGCC); *Xdkk-1* (f, ACAAGTACCAACCTCTG-GATGC; r, ACAGGGACACAAATCCGTTGC; 198-base parts); *mdkk-1* (f, TCTGCTAGGAGCCAGTGCC; r, GATGGTGATCTTCTGTATCC; 699 bp); *mdkk-2* (f, TGCCACAGTCCCCACCAAGGATC; r, CCTGATGGAG-CACTGGTTTGACG; 363 bp); *α-globin*²⁶; *CG13* (ref. 21); *HoxB9* (*Xlhb-6*)⁴⁵; *N-CAM*¹⁸; *Xenopus H4*, *Cer*, *Xbra*, *siamois*, *otx2*, *XAG1* and *actin* primers⁶; *chordin*, *Bmp-4* and *gsc* primers¹².

Antibodies. Anti-FLAG M2 affinity gel was from Kodak. Antibodies were raised in rabbits commercially (FZB Biotechnik, Berlin) against two synthetic *dkk-1*-derived peptides coupled to keyhole limpet haemocyanin and affinity purified using a standard protocol⁴⁶. Peptide sequences were: peptide 14, amino acids 237–255; peptide 15, 125–143. For metabolic labelling and immunoprecipitations, stage-VI oocytes were microinjected with 10 ng *dkk-1*-FLAG mRNA followed by overnight incubation in 1× Barth in presence of 0.1% BSA and 30 μCi ml⁻¹ ³⁵S-methionine. The medium was harvested and 5× RIPA (final 1× RIPA) and PMSF (final 1 mM) were added. Oocytes were homogenized in 13 μl per oocyte RIPA containing 10 mM EDTA, 0.1 mM L-methionine, 1 mM PMSF, and centrifuged for 4 min. The resulting supernatant and the medium were immunoprecipitated with anti-FLAG affinity gel followed by elution with 50 μg ml⁻¹ FLAG peptide in RIPA containing 1 μg ml⁻¹ BSA.

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Correspondence and requests for materials should be addressed to C.N. Coordinates of *Xenopus dkk* family members have been deposited in GenBank database with the following accession nos: *dkk-1*, AF030434; *mdkk-1*, AF030433; *hdkk-1*, AA207078, (5'–most clone); *mdkk-2*, AA265561; *hdkk-2*, W55979; *cdkk-3*, D26311; *hdkk-3*, AA324686 (5'–most clone).

Evidence for a subsurface ocean on Europa

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Ground-based spectroscopy of Jupiter's moon Europa, combined with gravity data, suggests that the satellite has an icy crust roughly 150 km thick and a rocky interior^{1–4}. In addition, images obtained by the Voyager spacecraft revealed that Europa's surface is crossed by numerous intersecting ridges and dark bands (called lineae) and is sparsely cratered, indicating that the terrain is probably significantly younger than that of Ganymede and Callisto⁵. It has been suggested that Europa's thin outer ice shell might be separated from the moon's silicate interior by a liquid water layer, delayed or prevented from freezing by tidal heating^{6–10}; in this model, the lineae could be explained by repetitive tidal deformation of the outer ice shell^{11–13}. However, observational confirmation of a subsurface ocean was largely frustrated by the low resolution (>2 km per pixel) of the Voyager images¹⁴. Here we present high-resolution (54 m per pixel) Galileo spacecraft images of Europa, in which we find evidence for mobile 'icebergs'. The detailed morphology of the terrain strongly supports the presence of liquid water at shallow depths below the surface, either today or at some time in the past. Moreover, lower-resolution observations of much larger regions suggest that the phenomena reported here are widespread.

The observations are of a region near 13° N, 273° W, just south of the intersection of two prominent ridges, Asterius Linea and Agava Linea (Fig. 1). These and similar ridges appear in Voyager images as bright lines with dark margins, so were called 'triple bands'. From Voyager data much of Europa's surface had been categorized as mottled terrain or plains¹⁵. The area discussed here is mostly mottled terrain. It is crossed by numerous triple bands mostly trending roughly southeast–northwest, and by discontinuous rays from the crater Pwyll, 700 km to the south. Nested images were taken at 1.2 km per pixel, 180 m per pixel and 54 m per pixel.

This terrain can be divided into four components: the general background plains, the lineae, locally disrupted areas, and the large disrupted area south of the main ridge crossing in Fig. 1. The background plains are composed of ridges superimposed on ridges,

so that the surface resembles that of a ball of string. The lineae are mostly long, linear bundles of ridges and furrows that stand at a higher elevation than the surrounding plains. Elevations derived from shadows and photometric measurements indicate that the youngest of the prominent ridges have elevations of 100–200 m. Older ridges have much more subdued relief. Our main concern here is with places where the plains are locally disrupted as seen in Fig. 2, and broad areas of disruption as seen in Fig. 1 and in detail in Fig. 3.

Around the periphery of the area seen in Fig. 1, the plains are locally disrupted to form quasi-circular spots which are generally darker than their surroundings. The spots are mostly 10–20 km across and appear to be local upwellings of some kind (Fig. 2). In some of the spots, the surface is simply raised to form a low, flat-topped dome on which the original texture of the surrounding plains is preserved. In more advanced states of development, parts or all of the original surface texture are replaced by a fine-scale blocky texture and the disrupted zone is bound by an inward-facing escarpment. The disruptions cut across ridges, even those ridges that are relatively young. Most of the spots have a lower albedo than the unaffected terrain, which gives the terrain a mottled appearance. The mottled terrain covers large areas of Europa, which suggests that the disruptive process just described is widespread.

To the south of the prominent ridge crossing in Fig. 1 is a dark, diamond-shaped area, 100 km across, where widespread disruption of the crust has occurred. Within the area the crust has broken into pieces up to 20 km across. The individual crustal blocks are flat-topped and surrounded by cliffs that are 100–200 m high, as indicated by shadows. Preserved on the surface of the blocks is the original plain's surface texture of criss-crossing ridges. The texture on many of the blocks enables the reconstruction of the

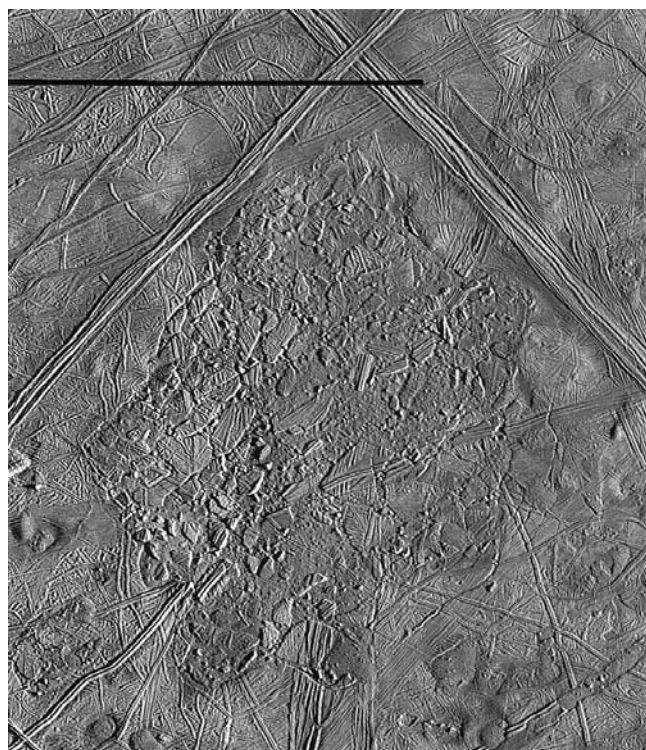


Figure 1 Disrupted zone at 13° N, 273° W. The scene is 200 km across. Just south of the prominent ridge crossing at the top of the image, the surface has been broken into blocks that have moved laterally from their original positions, as indicated by the texture of the pre-existing terrain preserved on the block surfaces. Around the periphery of the large disrupted zone are many additional, smaller and roughly circular areas where the original surface texture has been destroyed. Illumination is from the lower right.

original surface before its disruption. The reconstruction indicates that the blocks have moved laterally as much as several kilometres, and rotated. The area between the blocks has a much finer texture than the surface of the blocks. The texture resembles that in the locally disrupted areas described above. Some of the blocks appear to be tilted, thereby allowing the intervening hummocky material to lap onto one side of the block (Fig. 3). Other blocks are barely visible and appear to have been almost completely engulfed by the hummocky inter-block material. Whereas fracturing of the former surface has clearly formed the blocks, and the surfaces of the blocks themselves retain remnants of pre-existing fractures, fractures are rare in the intervening low-lying materials. The entire disrupted area is enclosed by an inward-facing cliff.

In the diamond-shaped disruption zone, lateral motion, rotation and tilting of sections of rigid icy shell have clearly taken place. As

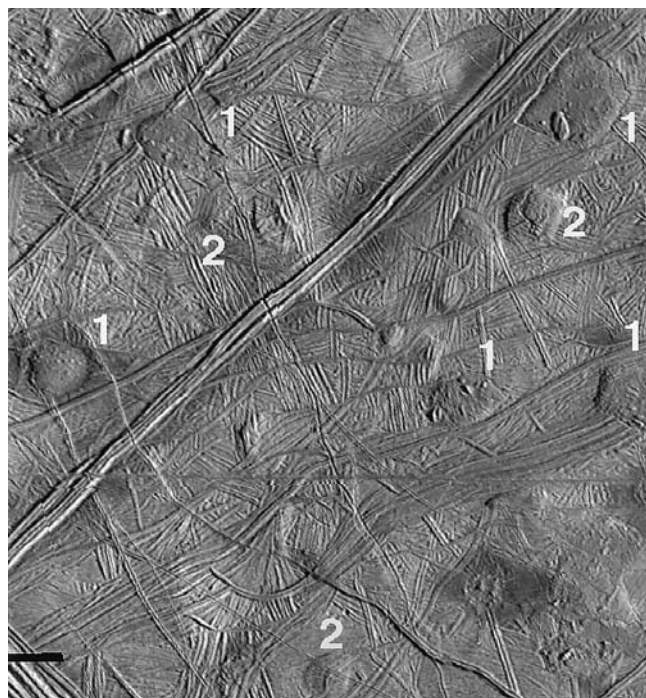


Figure 2 Detail from an area of mottled terrain 95 km across, just to the northeast of the area shown in Fig. 1. Most of the surface is composed of criss-crossing ridges and grooves. The features marked with a 1 are low domes on which almost all the ridge-and-groove surface texture has been destroyed. On domes marked with a 2 the original surface texture has been mostly preserved. The domes are thought to have been caused by columns of water or warm ice that erupted through the rigid ice shell¹⁶.



Figure 3 Detail of part of the lower left quadrant of Fig. 1. The scene is 40 km across. The terrain consists of high-standing blocks, on which the pre-existing surface texture is preserved, and low-lying terrain with a finer-scale texture, on which most of the features that characterize the original surface have been destroyed. Several small impact craters can be seen on the blocks, but none on the intervening terrain. Two prominent blocks in the right half of the picture are tilted to the south. The blocks are believed to be of the rigid ice shell and to have moved as a result of convection in the underlying water or warmer ice.

the surface is level, the lateral motion of the plates cannot be the result of downhill sliding but must rather be the result of movement of the materials in which the plates are embedded. As the plates moved, the underlying materials must have flowed upwards into the gaps that opened behind the plates, and sections of the ice crust must have foundered or been destroyed ahead of the plates. We have no model-independent measure of the thickness of the rigid shell, but when the breakup of the area being discussed here occurred, the thickness of the ice shell was probably comparable to or less than the horizontal dimensions of the smallest shell fragments that still retain their original surface texture. Thus the rigid shell was probably a few kilometres thick or less, and much thinner than the 150-km best estimate for the thickness of the whole water/ice layer.

We cannot tell from the images whether the breakup was relatively fast with the rigid plates floating in liquid water (which filled the opening gaps between the plates) or whether the process was slow, with the rigid plates overlying viscous ice that slowly filled the gaps. In the first case, the process would have been similar to the breakup of sea ice on the Earth¹⁶, and the 200-m height of the “icebergs” would imply that the plates were ~2 km thick. Some support for the water hypothesis is a “puddle” imaged in another area¹⁷ which suggests that an inviscid liquid (water) may have risen to the surface. In the alternative case where ice filled the gaps, water at relatively shallow depths may still be implied. Crude modelling of solid-state convection beneath a rigid crust on Europa suggests that effective viscosities of 10^8 – 10^9 MPa s, corresponding to an average temperature of 230–240 K (ref. 18), are probably needed. The presence of mobile ice below the rigid plates, at depths of several kilometres or less, implies local, near-surface thermal gradients of at least 10 K km^{-1} and temperatures approaching the melting point of ice at depths that are still very shallow compared with the 150-km thickness of the ice/water layer. Thus even with this alternative model, transient, modest increases in temperature could still result in melting, and water at shallow depths.

A number of observations suggest that the mobile layer (water or warm ice) is or was widespread, and not restricted to the small area discussed here. First, the quasi-circular spots that we now know to cause the mottling of at least half of Europa’s surface are probably the result of diapirs, that is, of columns of mobile material (water or ice) rising to the surface from some mobile layer below¹⁸. Second, rotation and lateral movement of large sections of Europa’s crust, and filling of the wedge-shaped gaps with materials darker than the surroundings, as found in several areas^{19–22}, also imply a mobile layer at depth. Last, the generally low surface relief despite abundant evidence of tectonic activity supports the presence of some accommodating, planet-wide mobile layer.

The time of formation of the various features described here is controversial. Whatever process caused the disruption, it took place at a later time than almost all other deformation. The paucity of

impact craters in all the images shown here is obvious. A number of small impact craters can be seen on top of some of the translated and rotated blocks in Fig. 3, but the low-lying material between the blocks is practically devoid of craters. From the latest estimate of the comet impact flux on Europa²³, and assuming plausible ratios of smaller comets to the observable larger ones²⁴, and that the observed craters are primaries, not secondaries, the age of the fractured surface is estimated to be of the order of 10^7 yr. With the same assumptions, the intervening materials are younger, perhaps a few million years old. Parts of the disrupted zone are overlain by rays from Pwyll crater and some of the craters appear to be Pwyll secondaries, not primaries. If this were taken into account, the derived ages would be even younger. As the uniquely prominent ray system of Pwyll suggests, it is the most recent large crater to have formed on Europa. Such craters are expected to form about every 10^6 yr. According to this model, even with the uncertainties in the cratering rates²³, it is difficult to see how the inter-block material, appearing so sparsely cratered at a resolution of 54 m per pixel, could be much older than 10^8 yr. (However, a minority of the authors of this Letter hold that the age of the surface can be better estimated by assuming that the youngest basin of Ganymede is 3.8×10^9 yr old, then deriving subsequent cratering rates from the number of craters superimposed on the basin and assuming a lunar decay constant for the cratering rate. This method yields a date of $\sim 3 \times 10^9$ yr for the area discussed here; G.N., manuscript in preparation). Although we cannot assert unequivocally that water is present today below the region shown in Fig. 1, if the young age of 10^8 yr—only a few per cent of the age of Europa—is correct, then the processes that have disrupted the plains are almost certainly continuing today, and liquid water is widely present at depths that are shallow compared with the 150-km estimated thickness of the water/ice layer. □

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Geological evidence for solid-state convection in Europa's ice shell

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The ice-rich surface of the jovian satellite Europa is sparsely cratered, suggesting that this moon might be geologically active today¹. Moreover, models of the satellite's interior indicate that tidal interactions with Jupiter might produce enough heat to maintain a subsurface liquid water layer^{2–5}. But the mechanisms of interior heat loss and resurfacing are currently unclear, as is the question of whether Europa has (or had at one time) a liquid water ocean^{6,7}. Here we report on the morphology and geological interpretation of distinct surface features—pits, domes and spots—discovered in high-resolution images of Europa obtained by the Galileo spacecraft. The features are interpreted as the surface manifestation of diapirs, relatively warm localized ice masses that have risen buoyantly through the subsurface. We find that the formation of the features can be explained by thermally induced solid-state convection within an ice shell, possibly overlying a liquid water layer. Our results are consistent with the possibility that Europa has a liquid water ocean beneath a surface layer of ice, but further tests and observations are needed to demonstrate this conclusively.

During its sixth orbit through the jovian system the Galileo spacecraft obtained high-resolution images (~ 70 and 180 m per pixel) covering a $\sim 10^5$ km² region of Europa centred near latitude 15° N, longitude 270° W. These images reveal that the satellite's late-stage geology is dominated by circular to elliptical pits, domes and dark spots ~ 7 – 15 km in diameter, spaced 5 – 20 km apart. These features generally modify and disrupt the pre-existing plains, which are widespread and are composed of subparallel ridges and grooves that overlap in successive generations¹. Lower-resolution images show that these features appear to constitute terrain like that of the previously defined “mottled plains” unit of Europa, which occurs over a large portion of the satellite's imaged surface⁶. Therefore, we infer that these pits, domes and spots are probably widespread across Europa, and are suspected to exist where mottled plains units occur.

In the region imaged at high resolution, a range of circular to elliptical features is observed with diverse specific morphologies (Fig. 1). The similarity in size and spacing of pits, domes and spots, and the gradation in morphology among them, suggests that they are genetically related. The features all have altered the original topography through positive or negative vertical deformation, and the vast majority are characterized by a surface morphology which is disrupted. The least-altered structures are characterized by a topo-

graphic rise with no disruption of background structure; somewhat more altered features include median fractures (Fig. 1a, b). The most disrupted features are characterized by a fragmented 'micro-chaos' region on top of the topographic highs, annular depressions, associated smooth low-albedo plains, and chaotic depressions which commonly destroy or embay the pre-existing background structural elements (Fig. 1c–f). Taken together, the structures appear to represent a sequence in which the surface was flexed upwards by intrusion beneath a relatively thin rigid surface layer, and experienced varying degrees of localized heating, collapse and extrusion.

This sequence is consistent with intrusion either by molten material, as would define a laccolithic intrusion, or by warm but relatively viscous solid-state material which rose buoyantly, through diapiric intrusion. We favour a diapiric origin of pits, domes and spots over laccolithic emplacement related to dyke intrusion, on the basis of the widespread occurrence, apparent spatial randomness relative to pre-existing structures, and general uniformity in diameter and spacing of these features. Based on the characteristics outlined in Fig. 1a–f, we propose a model in which diapirs of warm ice have risen from the deeper subsurface and have penetrated to within a few kilometres of the surface (Fig. 1g), that is, to a depth equivalent to about half the diameter of the zone of the most intense mechanical and thermal deformation. In some cases, rising diapirs are interpreted to have altered the surface topography and morphology by creating only a broad rise (Fig. 1g, left). In many cases, rising diapirs are interpreted to have affected more severely the surface and near surface (Fig. 1g, right), causing extension, deformation and possible sublimation and localized melting of the overlying surface, to produce 'micro-chaotic' terrain. The observed smooth, relatively low-albedo material (Fig. 1f) suggests thermal alteration of the surface⁸ or localized extrusion of melt, perhaps

produced locally if a warm ice diapir contacts a pocket of low-melting-point brine or ice⁹. The marginal moat commonly associated with pits and spots is possibly a "rim syncline"¹⁰ formed by downward flexing of the surface above the region from which rising diapiric material is withdrawn.

Diapirism can be triggered by compositional layering, producing density inversions and gravitational instabilities, as in regional salt layers underlying denser rocks on Earth¹¹ and as suggested for pits on Triton¹². Diapirism also can be induced thermally by means of solid-state convection, which may occur within any icy body if a sufficient thermal gradient exists, especially if the temperature at the base of the ice layer is near the solidus¹³. Models for the internal structure of Europa suggest that tidal heating might be sufficient to maintain a liquid water ocean ~10–30 km beneath its surface, its modelled depth varying with latitude and longitude^{2–5}. Reynolds and Cassen¹⁴ have investigated a model in which a liquid water layer might drive solid-state convection within a large icy satellite, such as Europa. Here we re-evaluate this solid-state convection model, using recently updated parameters describing the flow of ice in which strain rate is a function of stress, temperature and ice grain size^{15,16}.

Convection in an icy satellite would be expected to occur in a warmer sublayer beneath a cold brittle surface lid. The critical Rayleigh number Ra describing the onset of convection is related to the thickness h of the sublayer and the temperature difference ΔT across it as:

$$Ra = \rho g h^3 \alpha \Delta T / (\kappa \eta). \quad (1)$$

Here ρ is density, α is thermal expansivity, κ is thermal diffusivity and η is viscosity. We choose $Ra \approx 2,000$ as appropriate to a variable-viscosity fluid¹⁷ and consider the range 500–3,500 to cover uncertainty in this value. Gravity g is 1.31 m s^{-2} for Europa.

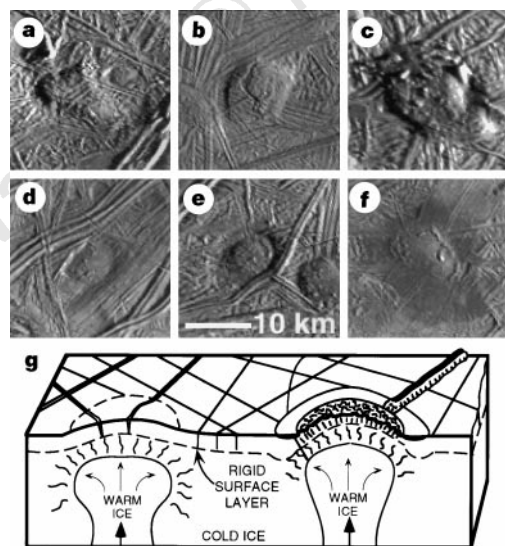


Figure 1 Pits, domes and spots of various specific morphologies (shown to a common scale) on the surface of Europa. North is to the top and illumination is from the east. **a**, A dome which has flexed upwards pre-existing plains material without disrupting it; **b**, a dome with a median fracture; **c**, a disrupted plateau, suggesting a combination of uplift and disruption of material; **d**, a depression with disrupted 'micro-chaos' material; **e**, a disrupted plateau with a depressed annulus; and **f**, a disrupted area with an annulus of dark smooth material. **g**, Block diagram illustrating the interpretation of these features as due to diapiric rise of warm ice, and mechanical and thermal alteration of the surface and near surface.

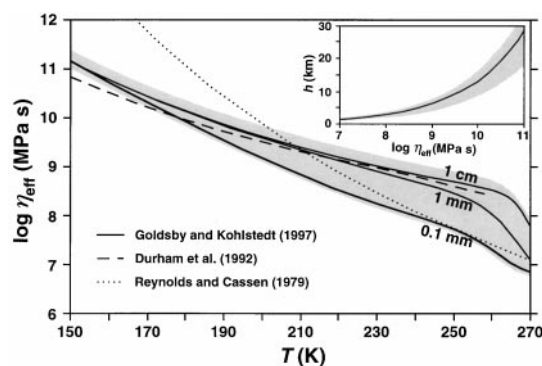


Figure 2 Effective viscosity η_{eff} of ice as a function of temperature T , strain rate ϵ , and grain size d for conditions relevant to the subsurface of Europa. Solid lines represent a grain-size-sensitive flow law for ice^{15,16} (extrapolated to lower temperatures from the experimental conditions of $173 \text{ K} \leq T \leq 248 \text{ K}$, and extended to warmer temperatures on the basis of unpublished data of D.L.G. and D. L. Kohlstedt), for a nominal Europa strain rate⁵ of $\epsilon = 2 \times 10^{-10} \text{ s}^{-1}$, and varying with grain size as labelled. The shaded region represents η_{eff} for a likely range in European subsurface conditions, from $\epsilon = 1 \times 10^{-10} \text{ s}^{-1}$ and $d = 1 \text{ cm}$ (upper bound of shaded region) to $\epsilon = 3 \times 10^{-10} \text{ s}^{-1}$ and $d = 0.1 \text{ mm}$ (lower bound). At relatively warm temperatures, η_{eff} varies by an order of magnitude over the range of conditions considered. The dotted line represents the temperature-dependent relationship for ice viscosity adopted by Reynolds and Cassen¹⁴, and the dashed line represents the power-law (grain-size independent) ice flow relationship of Durham *et al.*²⁹ (derived over the temperature range $195 \text{ K} \leq T \leq 258 \text{ K}$ and extrapolated here to lower temperatures). Inset, Critical layer thickness h as a function of η_{eff} for the onset of convection on Europa as predicted by equation (1). Solid line indicates the nominal case of $Ra = 2,000$; upper and lower bounds of the shaded region correspond to $Ra = 3,500$ and 500 , respectively.

Parameter values for ice are evaluated at the average temperature appropriate to the potentially convecting sublayer, estimated as¹⁸

$$T_b = \{(Q/4R) - [(Q/4R)^2 - QT_i/R]^{1/2}\} - T_i \quad (2)$$

where Q is the activation energy for ice creep, R is the gas constant, T_b is the sublayer's lower boundary temperature (assumed to be ~ 273 K), and T_i is the upper boundary temperature. We find that grain boundary sliding ($Q = 49$ kJ mol⁻¹)^{15,16} dominates the deformation of ice over the range of potential average sublayer temperatures, for the grain sizes and stresses modelled for Europa (see below), giving $T_i = 197$ K and $\Delta T = 76$ K. For ice¹⁹ at $T_{ave} = 235$ K: density $\rho = 923$ kg m⁻³, thermal expansivity $\alpha = 1.4 \times 10^{-4}$ K⁻¹, and thermal diffusivity $\kappa = 1.4 \times 10^{-6}$ m² s⁻¹.

Previous analyses of solid-state convection on icy satellites^{14,20} have assumed a strictly temperature-dependent viscosity for ice. The effective viscosity η_{eff} of a non-newtonian material such as ice is more accurately defined as

$$\eta_{eff} = \sigma/(3\dot{\epsilon}_{tot}) \quad (3)$$

where either stress σ or strain rate $\dot{\epsilon}$ is assumed fixed. Several processes may deform Europa's ice shell on a long timescale ($>10^5$ yr) including non-synchronous rotation^{21,22}, polar wander²³, and viscous flow from thick towards thin portions of an ice shell²³. However, far more rapid straining is induced by tidal deformation as the satellite travels through its 3.6-day elliptical orbit²¹. This strain rate is modelled as $(1-3) \times 10^{-10}$ s⁻¹, varying with latitude and longitude⁵, and will control the effective viscosity of Europa's subsurface ice as a function of its grain size (Fig. 2). The grain size of Europa's relatively pristine surface plains inferred from Galileo imaging²⁴ is ~ 0.1 mm, and the range chosen here represents a possible increase with depth, as through sintering.

At $T_{ave} \approx 235$ K, the range of strain rates and grain sizes considered predicts $\eta_{eff} \sim 10^8-10^9$ MPa s, and corresponding stresses $\sim 0.1-0.4$ MPa. From equation (1), the corresponding thickness of the sublayer when convection initiates is $\sim 3-7$ km for $Ra \approx 2,000$, and $\sim 2-8$ km for the range of Ra considered (Fig. 2, inset). The onset of convection should vary across Europa, depending on local heat flow, strain rate and ice grain size. For finite-amplitude convection, the spacing of upwelling regions is predicted to be about twice the sublayer thickness, nominally $\sim 6-14$ km for Europa; although the convective state in Europa's ice shell may not be related to the linear state at the onset of convection, this dimension is remarkably similar to the $\sim 5-20$ km spacing of Europa's domes, pits and spots.

The thickness of Europa's whole ice shell when convection initiated can be estimated if the thickness of the overlying rheological lithosphere is known. The degree of thermal and mechanical alteration revealed by pits and micro-chaos regions suggests intrusion of warm diapirs to a few kilometres depth or less; moreover, a variety of geological analyses suggest that the thickness of Europa's overlying brittle lithosphere was probably ≤ 2 km thick^{1,25,26} at the time of its deformation. This suggests a total ice shell thickness of $\sim 3-10$ km. This is significantly less than the 30 km value of Reynolds and Cassen¹⁴, who derived an upper-limit shell thickness of initiation of convection, and who used a strictly temperature-dependent viscosity relationship. Our value is also less than the $\sim 10-30$ km whole-shell thickness predicted by thermal modelling⁵, which assumes the absence of convection. We estimate the vertical scale of Europa's upward domes as <100 m, based on comparison with nearby features that cast shadows; a rise of warm ice through a $\sim 3-10$ km ice shell to a level of neutral buoyancy is consistent with this inferred topography, though the precise buoyancy forces would be affected locally by the presence of salts, the topography of the warm and cold ice layers, and the rise and cooling rates of diapirs.

Overall, we find strong similarity between the morphology, geometry and spacing of surface features plausibly interpreted as due to diapirism, and revised predictions for the onset of convection

in a floating ice shell on Europa. If a european shell cooled and thickened above a liquid water interior to the point where solid-state convection initiated regionally, the geological style may have changed from widespread tectonism associated with a thin ice shell to vertical deformation linked to diapirism. This is consistent with the stratigraphic history observed until now, in that pits, domes and spots consistently disrupt linear features but are rarely cut by them. Examples of domes, pits and spots surrounding, and apparently within, a large area of chaos¹ imaged by Galileo suggest that this chaos area may represent a region of intense diapiric upwelling, perhaps related to local variations in heat flux, instability development, or thickness of the rigid surface layer, and so does not provide certain evidence of melting.

Solid-state convection is an efficient heat loss mechanism, predicted to freeze a european ocean² in $\sim 10^8$ yr, unless sufficient heat is continually supplied to the convecting ice, for example through tidal heating. Indeed, because tidal dissipation is greatest in the warmest portion of an ice shell⁵, tidal dissipation may be enhanced in a floating ice shell warmed throughout by convection, perhaps allowing an ocean to be maintained²⁷.

Although gravity measurements suggest that Europa is differentiated, with an outer H₂O layer ~ 150 km thick²⁸, its state (completely solid, or a solid shell above liquid) is not directly addressed by the gravity data. Models for Europa diapirism could be proposed that do not require a liquid layer, for example solid-state convection of a thick ice layer with convection driven by a steep thermal gradient, or diapiric instability driven by compositional layering; however, explicit modelling of such schemes is necessary to evaluate their consistency with the observations and interpretations we have described. We have shown that the geological observations and implications of pits, domes and spots are consistent with a model of diapirism as representative of convection in a floating ice shell. In combination with the low crater density and inferred young surface age of Europa¹, this increases the likelihood that liquid water may persist to the present day beneath the surface of Europa. Continuing Galileo imaging and morphological analyses, along with additional modelling of Europa's internal configuration and heat balance, will further test this idea. $\square$

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Evidence for non-synchronous rotation of Europa

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Non-synchronous rotation of Europa was predicted on theoretical grounds¹, by considering the orbitally averaged torque exerted by Jupiter on the satellite's tidal bulges. If Europa's orbit were circular, or the satellite were comprised of a frictionless fluid without tidal dissipation, this torque would average to zero. However, Europa has a small forced eccentricity $e \approx 0.01$ (ref. 2), generated by its dynamical interaction with Io and Ganymede, which should cause the equilibrium spin rate of the satellite to be slightly faster than synchronous. Recent gravity data³ suggest that there may be a permanent asymmetry in Europa's interior mass distribution which is large enough to offset the tidal torque; hence, if non-synchronous rotation is observed, the surface is probably decoupled from the interior by a subsurface layer of liquid⁴ or ductile ice¹. Non-synchronous rotation was invoked to explain Europa's global system of lineaments and an equatorial region of rifting seen in Voyager images^{5,6}. Here we report an analysis of the orientation and distribution of these surface features, based on initial observations made by the Galileo spacecraft. We find evidence that Europa spins faster than the synchronous rate (or did so in the past), consistent with the possibility of a global subsurface ocean.

Several testable predictions can be based on the hypothesis of non-synchronous rotation of Europa. First, systematic changes in the orientation of lineaments with age may be expected as the surface reorients relative to fixed global patterns of tidal stress. Second, crustal extension is predicted in the regions west of the tidal bulges as the surface stretches to accommodate the changing shape. Third, no asymmetry in impact crater density between leading and trailing hemispheres should be found if Europa's surface has rotated continually with respect to Jupiter. Last, if the rotation rate is large

enough, the locations of surface features seen in Galileo images might be displaced eastwards relative to their positions in Voyager data. Galileo Solid State Imaging (SSI) data represent a substantial improvement over Voyager data in terms of spatial resolution, spatial coverage and spectral range of the observations. After four close passes of Europa and several non-targeted encounters⁷, samples of the surface at various locations have been imaged at resolutions down to 20 m per pixel; coverage has been expanded especially on the trailing hemisphere, and observations of Europa's surface at near-infrared wavelengths have revealed lineaments invisible to Voyager bandpass filters.

Galileo colour data provide one test of non-synchronous rotation. Multispectral imaging of Europa's northern high-latitude region was performed during Galileo's first orbit of Jupiter⁷. The imaged region extends across the trailing side of the anti-jovian hemisphere, centred at 45°N, 221°W. False-colour composites made up from these images show at least three distinct classes of linear features on Europa's surface (Fig. 1). These features may represent different stages of development of tectonic lineaments on Europa. Their distributions are shown in Fig. 2, derived from photogeological and spectral mapping (supervised classification) of the photometrically corrected four-colour data. Bands with spectral reflectance similar to the bright wedge (Fig. 2a) make up the stratigraphically oldest lineaments and generally have south-west–north-east trends. The intermediate-aged triple-bands (Fig. 2b) trend roughly east–west, with the younger of the two

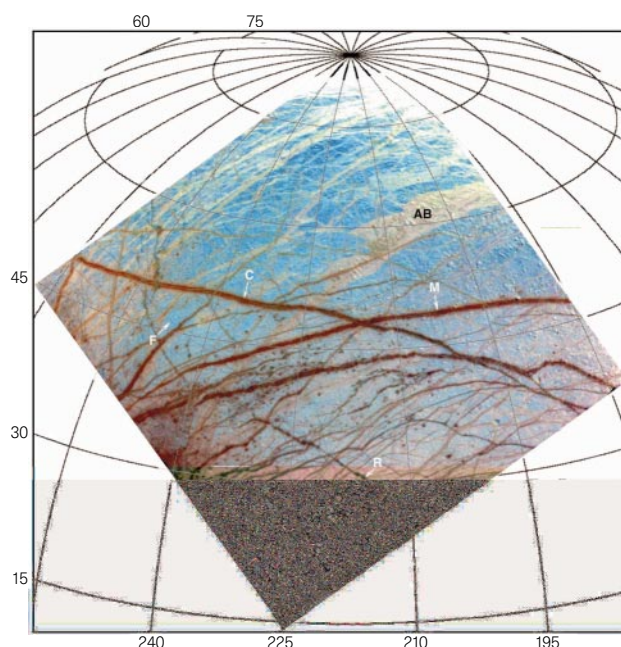


Figure 1 False-colour composite of northern high-latitude region of Europa, produced from images taken through the 968-nm, 756-nm and green filters. Overlaid is a grid showing location in degrees North latitude and West longitude. The most prominent linear features are the dark triple-bands such as Cadmus Linea and Minos Linea. The triple-bands overprint many older lineaments which are intermediate in colour between the triple-bands and the icy plains, but are brighter than the triple-bands and the surrounding icy plains at near-infrared wavelengths. These older lineaments include a bright wedge at 60°N, 200°W, which is somewhat similar to the grey bands identified in the southern polar regions in Voyager images¹⁴. The youngest features appear to be incipient fractures, less than a pixel (1.6 km) wide, which cross-cut the dark triple bands. Labelled on this figure, in order of age (as determined from cross-cutting relationships), are young fractures (F), developing band Rhadamanthys Linea (R), intermediate-aged triple bands Cadmus Linea (C) and Minos Linea (M), and a wedge-shaped ancient band (AB).

prominent criss-crossing bands near the centre of the image (Cadmus Linea, labelled '2') having the more northwesterly trend. Rhadamanthys Linea ('R'), presumably a triple-band in the early stages of formation⁷, has spectral characteristics similar to fully developed triple bands but has an orientation closer to that of the youngest fractures (Fig. 2c), which run northwest–southeast. The morphology of Rhadamanthys is transitional between the young fractures and the triple-bands, suggesting that these features are all formed by the same tectonic processes. If so, then a clockwise rotation of stress direction has taken place in this region over time.

We suggest that the explanation for the reorientation of these lineaments is non-synchronous planetary rotation. As Europa's surface revolves relative to the tidal figure, the pattern of stresses experienced in the northern hemisphere should rotate in a clockwise sense, consistent with the observations. This is equally true whether the fractures are produced by the long-term stresses induced by non-synchronous rotation or by much shorter-timescale tidal distortions continually reorientated by the non-synchronous rotation. Polar wandering, in contrast, could not produce the observed reorientation⁸. An estimate of the amount of eastward rotation implied by the observations can be obtained by matching the orientations of the lineaments to theoretical biaxial stress trajectories, shifted in the longitude direction until the lineaments are perpendicular to the maximum tensional stress. About 60° of eastward reorientation is required to explain the rotation inferred between the oldest lineaments (Fig. 2a) and the younger of the two prominent intermediate-aged triple bands, Cadmus Linea ('2' in Fig. 2b). A further rotation of more than 30° is required if the youngest fractures (Fig. 2c) formed by the same process.

The above analysis does not assume that Europa's lineaments necessarily formed in response to the stresses associated with rotational reorientation, but merely that they were carried along as the surface subsequently shifted eastwards. In fact, the orientation of the youngest fractures (incipient cracks, along with Rhadamanthys Linea) is roughly radial to the anti-jove point (Fig. 2c). This is not consistent with the stress pattern predicted for non-synchronous rotation, which is displaced 45° to the east⁵, but instead fits the current tidal stress regime. These youngest lineaments are apparently produced by diurnal tidal stresses due to Europa's forced eccentricity, and shifted eastwards by non-synchronous rotation over much longer timescales. Tidal flexing is predicted to produce periodic bending and tensional stresses radial and concentric to the sub- and anti-jove points, along with longitudinal oscillations in the orientation of the tidal bulges⁹. Extension along small circles concentric to the anti-jove point occurs during half of

each 3.6-day orbit, as Europa recedes from Jupiter and the tidal bulges relax. Fractures radial to the anti-jove point may form as the ice fails in tension, perhaps incrementally over many thousands of orbits. Tidal flexing produces stresses which alternate in sign each european 'day', providing a possible mechanism for ridge formation by repeatedly opening and closing the cracks and pumping icy material from Europa's interior to the flanks of the fractures. Ridge-building is the dominant process shaping this moon's surface, and every landscape that has escaped erasure by heating from below^{10,11} is imprinted with generation after generation of intersecting ridges with widely varying scales and orientations. The constant reorientation of the tidal stress field owing to non-synchronous rotation should produce continual migration of the local stress directions over time, explaining the observed sequential changes in ridge direction with stratigraphic age. If so, then the multiple generations of lineae seen in high-resolution images of Europa seem to suggest that the process has gone on for many rotational cycles over the age of the surface.

A second test of non-synchronous rotation was provided by Galileo imaging of the trailing hemisphere of Europa. Concentrations of tensional stresses west of the sub- and anti-jove points are expected from non-synchronous rotation, as the surface stretches over the tidal bulges. A large region near Europa's equator to the west of the anti-jove point is characterized by relatively short, dark wedge-shaped bands which are morphologically distinct from the global lineaments discussed above. From Voyager observations, these bands were believed to be due to crustal extension on the basis of their polygonal boundaries¹² and the fact that the brighter pre-existing crustal plates could be reconstructed by rotation and translation with little loss or overlap¹³. The region was only partially imaged by Voyager, however, leaving uncertain the cause of the extension; it had been mapped^{12–14} at longitudes ranging from 170° to 220° W. With the more complete coverage from Galileo, the zone is now known to be centred near 210° W, significantly displaced from the apex of tidal distortions, and to extend westwards to at least 240° W.

The tensional stresses expected for an incremental eastward rotation of the surface are centred 45° west of the tidal axis, with corresponding compression to the east⁵ (no tectonic features indicative of compression have been recognized to the east of the anti-jove point, perhaps because ice is stronger in compression than it is in tension). Adding to this are the diurnal stress components due to libration that tug the tidal bulges westward during the half-orbit centred above apojove. The distribution of dark wedges (Fig. 3) matches the predicted extent of tensional stresses in longitude,

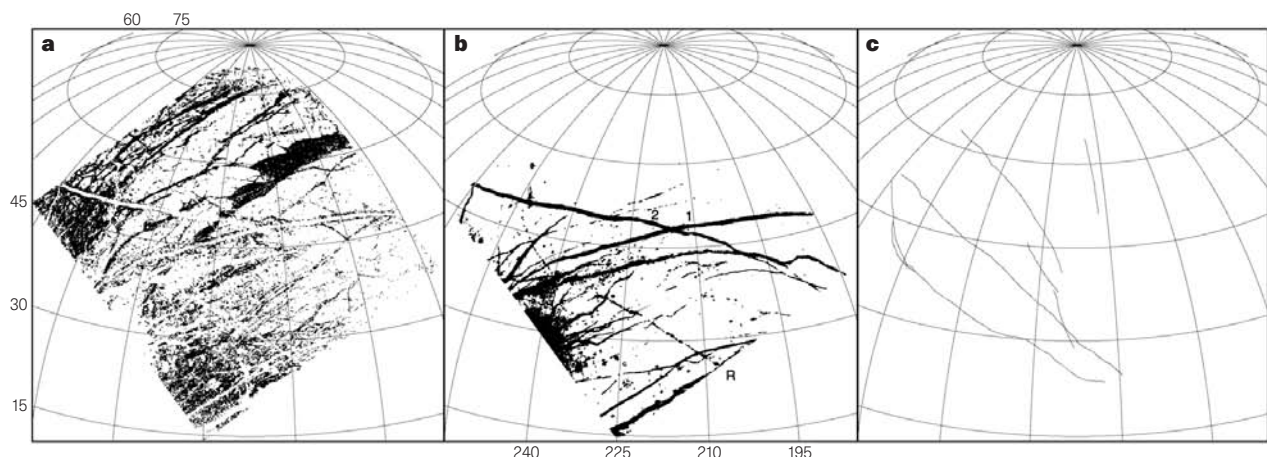


Figure 2 Distribution of ancient bands and bright wedges (a), intermediate-aged triple-bands and similarly coloured materials (b), and young fractures (c) which cross-cut the triple bands. In b, '1' and '2' refer to the order of placement of the two

prominent triple bands Minos Linea and Cadmus Linea near the centre of the scene, while 'R' marks Rhadamanthys Linea, a feature evidently transitional between the young fractures and the fully developed triple-bands.

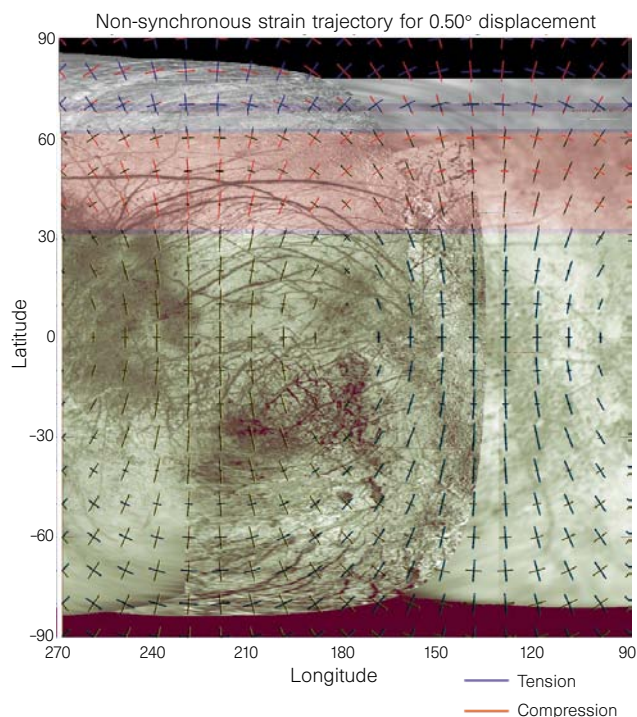


Figure 3 Principal stress directions for an incremental eastward shift in the orientation of the surface with respect to the tidal figure of Europa (coloured lines), overlain on a combined Galileo/Voyager mosaic of the anti-jovian hemisphere. The maximum tensional stresses (blue) are reached in the region 45° to the west of the anti-jove point, where both of the horizontal principal stresses are tensile. A rift zone centred at 15° S, 210° W may have formed as the surface stretched over the tidal bulge. We note that the principal stress directions rotate clockwise towards the east at high northern latitudes, but rotate in an anticlockwise sense in the southern hemisphere.

especially if allowance is made for eastward rotation after formation. In particular, the region closely corresponds to the zone in which both principal horizontal stresses are tensile (shown blue in Fig. 3). However, the pull-apart zone appears offset from the equator, centred at a latitude of ~15° S. This displacement is not predicted by non-synchronous rotation, but may be due to polar wander (rotation about an axis through the sub- and anti-jove points¹⁵) or to irregularities in the interior of Europa. High-resolution images of the dark wedge-shaped bands suggest that the rifting occurred episodically in both the northeast–southwest and northwest–southeast directions, along with local rotation and translation of the rigid pre-existing crustal blocks¹⁶.

The two remaining predictions of non-synchronous rotation can be summarized succinctly. No asymmetry in impact crater density between leading and trailing hemispheres should be found if Europa's surface has continually rotated with respect to Jupiter; indeed, imaging observations obtained during Galileo's seventh orbit show no apparent excess of impact craters at the apex of the satellite's leading surface. No discernible change took place in the positions of surface features with respect to the terminator between the Voyager and Galileo eras after allowance was made for synchronous rotation, allowing us to place a lower limit of 10^4 yr on the rotation period of Europa with respect to Jupiter¹⁷.

Confirmation of non-synchronous rotation of Europa's surface may have important implications for the structure of the satellite's interior. Anderson *et al.*³ postulated significant non-hydrostatic components to the gravity field of Europa to account for discordant results obtained from Doppler data during Galileo's first two close fly-bys of the satellite. Although it is not possible to uniquely determine moments of inertia from the gravity field alone, the departures from hydrostatic equilibrium are inferred to be comparable to the gravitational effects of the tidal bulges. For comparison, a difference in the equatorial moments of inertia $(B - A)/C$ only 10^{-2} to 10^{-3} of the hydrostatic value (due to the equilibrium tidal bulges) would be sufficient to lock Europa into synchronous rotation¹. If Europa's interior possesses a sufficient mass distribution asymmetry to tidally lock the moon towards Jupiter—as seems indicated by the gravity results—and yet the surface shows evidence of asynchronous rotation, then the crust is required to be mechanically

decoupled from the interior. Such decoupling could be produced by a subsurface ocean or possibly a layer of ductile ice.

The hypothesis of non-synchronous rotation will be further tested by moderate-resolution global colour imaging during the extended mission of Galileo. We predict that lineaments will rotate in an anticlockwise sense in the southern hemisphere. We also expect that a corresponding equatorial rift zone, similar to the region of dark wedge-shaped bands, should be found to the west of the sub-Jupiter point, an area not yet seen by Galileo. Unfortunately the Jupiter-facing hemisphere will be the last to be imaged by the spacecraft, in November 1999. □

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Episodic plate separation and fracture infill on the surface of Europa

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Images obtained by the Voyager spacecraft revealed dark, wedge-shaped bands on Europa that were interpreted as evidence that surface plates, 50–100 km across, moved and rotated relative to each other¹. This implied that they may be mechanically decoupled from the interior by a layer of warm ice or liquid water^{2,3}. Here we report similar features seen in higher resolution images (420 metres per pixel) obtained by the Galileo spacecraft that reveal new details of wedge-band formation. In particular, the interior of one dark band shows bilateral symmetry of parallel lineaments and pit complexes which indicates that plate separation occurred in discrete episodes from a central axis. The images also show that this style of tectonic activity involved plates < 10 km across. Although this tectonic style superficially resembles aspects of similar activity on Earth, such as sea-floor spreading and the formation of ice leads in polar seas, there are significant differences in the underlying physical mechanisms: the wedge-shaped bands on Europa most probably formed when lower material (ice or water) rose to fill the fractures that widened in response to regional surface stresses.

Images of Europa obtained during the first and fourth orbits of the Galileo spacecraft around Jupiter show that a zone of dark, wedge-shaped bands seen obliquely in limited 2 km per pixel

Voyager coverage extends WSW to about 40° S, 250° W in a prominent belt, 2,000 km by 600 km (Fig. 1). The zone is centred at about 15° S, 215° W, southwest of the anti-Jupiter point. Dark bands within this disrupted zone have other characteristic shapes besides wedges, including narrow crescents and trapezoids with maximum band widths of ~35 km. Contacts between dark band materials and surrounding brighter plains generally appear sharp at a resolution of 1.2–1.6 km per pixel (unlike the diffuse boundaries of triple bands revealed at the same resolution⁴). Sharply defined opposing margins of each dark band match closely in many instances, indicating that separation, translation and rotation (generally <10°) of icy plates occurred across areas now filled with dark band materials. This confirms, and expands to new areas of Europa, the conclusions of Schenk and McKinnon² that prominent dark bands making sharp contacts with brighter materials represent expressions of surface extension and plate separation.

A 420 m per pixel image obtained on orbit 3 reveals details of a prominent dark wedge band seen in Voyager 2 coverage. At Voyager resolution, dark wedge bands were reconstructed using single rotational or translational vectors to close each feature^{2,3}. Higher-resolution Galileo data reveal that these features are much more complex, with each band involving many smaller plates moving individually or in groups (Fig. 2). Reconstruction across the most prominent dark band in the 420 m per pixel image, at 17° S, 197° W, cannot be accomplished using a single rotational or translational vector, but involves movement of at least 20 individual plates of bright plains material in a relatively small area. The northern 75 km of the band can be closed by ~18 km of ENE–WSW translation. The southern 105 km of the band can be closed by rotations of ~9° around a pole located at 21° S, 195° W. Two other, older bands, each with parallel lineaments in their interiors, can be closed with average plate translations of ~17 and 20 km. Cross-cutting relationships among these three dark bands indicate brightening to background terrain levels with increasing age, consistent with age/brightness trends of triple bands⁴ and with the regional reconstruction of plates and stratigraphy in this area by Tufts *et al.*⁵. The two older bands could not be detected easily or evaluated using Voyager data, but clearly they contribute significantly to the structural history of the region, especially to the overall direction and amount of regional extension. Reconstruction of bright plates into pre-fractured configurations by dispensing with intervening dark material can be accomplished with little to no overlap of plate margins (Fig. 2), but a small fraction of bright plains material is missing and has somehow been consumed, or covered by or altered to darker materials. The small size of the plates allows the existence of viscous or liquid materials closer beneath the surface (at the time of plate movement) than would be suggested by the smallest plates visible at Voyager resolution. Many internal details are visible in the youngest of the three large dark bands, including: (1) interior lineaments that

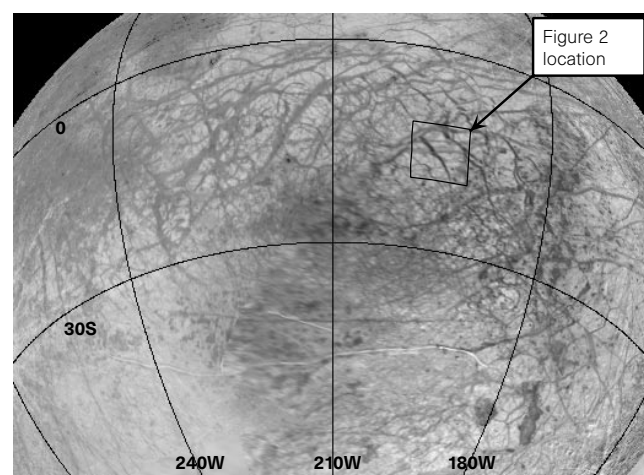


Figure 1 Combined Voyager and Galileo image data reprojected from above 40° S, 205° W. The location of the 420 m per pixel image in Fig. 2 is indicated. The 2,000-km belt of prominent dark, wedge-shaped bands extends in a south-facing crescent across the upper part of the figure. Bright plains marked with wedge-shaped bands partially surround a densely fractured region centred at 40° S, 205° W. Scale: 30° of latitude = 820 km.

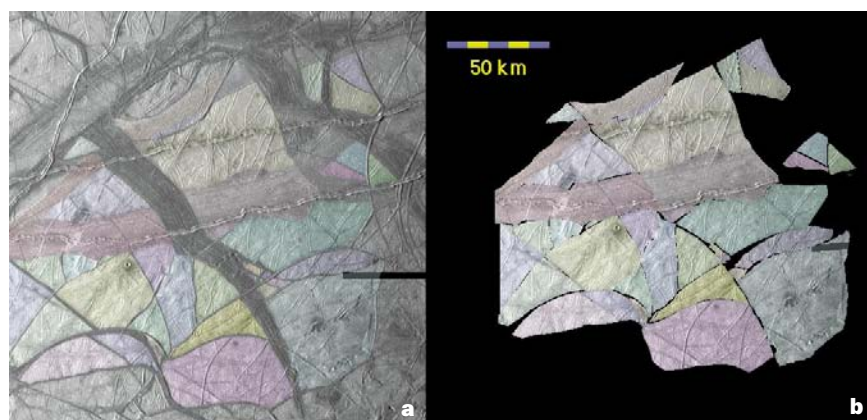


Figure 2 Part of Galileo image S0368639400 of Europa, with north up. In this area plates of bright plains materials, some <10 km across, can be fitted together with no overlap by dispensing with intervening dark materials. Twenty individual bright plates involved in closing the prominent dark, wedge-shaped band in the centre of **a** have been tinted to demonstrate reconstruction with no plate overlap in **b**.

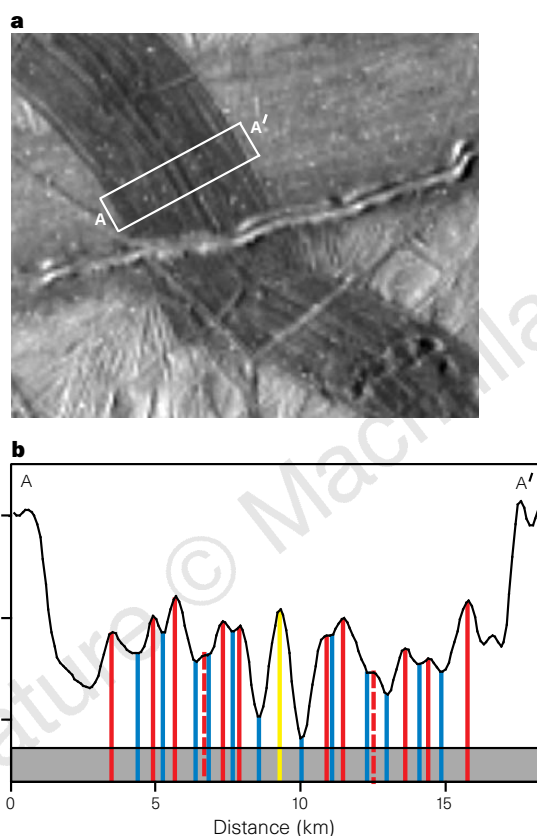


Figure 3 Bilateral symmetry of internal details within the dark, wedge-shaped band shown in Fig. 2. Enlargement (with interpolation/smoothing) in **a** of the view in Fig. 2 shows parallel internal lineaments and mirrored arrangements of four pits (southeast) in the dark band. An averaged brightness profile in units of image data numbers (DN) from A to A' is shown in **b**, where the locations of convex-up and concave-up inflections are marked in red and blue, respectively. Minor inflections (dashed) are less certain. The order of brightness inflections in the grey strip along the bottom of the plot is symmetric around the central axis of the band, marked by the yellow line.

parallel band margins and each other; (2) a central lineament pair; and (3) bilateral symmetry of some features, including prominent internal lineaments, brightness inflections, and pit complexes (Fig. 3). Narrower dark bands seen in the image lack many or all of these characteristics at available resolution, and have neutral or positive relief.

Bilateral symmetry of some features within the youngest dark band is consistent with repeated emplacement of materials primar-

ily at the centre of the band, with material moving to each side to make way for newer material rising from below as the bright plates moved further apart. Lineaments closer to the centre of the band trace a more rounded version of the band margins, indicating intervals of inactivity long enough for structural adjustments to occur which affected the trend of succeeding spreading episodes. Internal ridges and troughs (and more subtle brightness inflections that are probably due to poorly resolved ridge and trough topography) indicate that dark materials were emplaced at the centre of the band with substantial viscosity, or became highly viscous rapidly enough after emplacement to retain structure while migrating from the band axis. Consistent with this are the much narrower bands of dark material in the 420 m per pixel image that stand tens of metres above the bright plates they divide, indicating they were extruded too viscously to flow onto surrounding terrain. It is unclear from the image whether or not deeper material rose in a viscous state as well. (If it did, viscosity of material rising towards the central axis of the band could gradually have imposed a slightly more efficient conduit configuration with a straighter trend between the band margins, resulting in the observed rounding of the trend of the band axis with time.) An alternative terrestrial sea-ice analogy involving repeated opening and refreezing of 'leads' of liquid water must still account for the incremental emplacement of dark material in discrete episodes along a central axis, and in this model progressive straightening of the axis with time is harder to explain. These issues can only be settled when very high-resolution images are obtained over this feature later in the Galileo mission; but in any case, the dark band materials are shown to be capable of plastic flow around very small bright plates, only a few kilometres across, that remain undeformed by the process. This implies that deformable material, such as plastically deforming ice or liquid water, was present at depths on the same scale as the smallest plate dimensions. The small percentage of missing (or darkened) bright plate material in the reconstruction contrasts with areas of chaos on Europa such as Conamara, where breakup of icy crust into mobile plates occurred with significant collateral destruction of plate material (perhaps including subsidence and burial) and replacement by a chaotic matrix of icy rubble that might have been emplaced fluidly⁶. In those regions crust was destroyed and/or buried during resurfacing, while in the 2,000-km belt of dark bands crust was created seemingly to fill gaps that opened between preserved, undeformed plates.

Dark bands with matching sharp margins provide good evidence for crustal extension in these areas, but so far there is no clear evidence of corresponding zones where bright plate material was consumed with the growth of dark bands. The close fit obtained when bright plates are reassembled implies that their shapes and surface areas remained relatively constant during spreading, so features analogous to terrestrial subduction zones should not be expected within the bright plates. The possibility that dark bands might at times alternate as sites of convergence, closure, and

consumption of surface materials cannot be ruled out, but the apparent lack of convergence zones is one of several important differences between plate tectonics on Earth and what has been seen so far of plate movement and dark band growth on Europa.

Even without surface signatures of plate convergence, could the ~ 100 -km spacing of the more prominent dark bands (Fig. 1) be a result of subsurface convection that does not, or did not, involve recycling of surface materials? If so, this would be on a different scale to solid-state convection proposed near Conamara Chaos⁷. In that area Pappalardo *et al.*⁷ conclude that domes, dark spots, and pits are evidence for thermally driven vertical movements 7–15 km in diameter and 5–20 km apart in the near-subsurface. Domes, dark spots, and pits are much less pervasive among the dark wedge bands discussed here, suggesting that heat transfer from the interior occurred differently in this area. Although a water-ice-rich layer > 50 km thick is allowed by several analyses that assume differentiation and segregation of water from a deeper silicate mantle^{8,9}, as well as interior models based on gravitational data derived from Doppler tracking of the Galileo spacecraft¹⁰, the idea that these materials are organized into a network of ~ 100 -km convection cells causing dark band formation is inconsistent with the existence of isolated dark wedge bands (for example, near 52° N, 230° W).

A more likely explanation is that plate fracturing and movement in response to regional surface stresses simply allowed material to rise passively to the surface to form the dark bands. A very densely fractured region centred at 40° S, 205° W is partly surrounded by bright plains with prominent dark bands showing evidence of plate offsets^{1–3,11–13} (Fig. 1). This annulus includes the disrupted zone seen by Galileo on its first and fourth orbits and the dark bands seen at higher resolution on the third orbit. Destruction of surface material in the area of 40° S, 205° W could have provided room in surrounding terrain for icy plates to separate and rotate relative to one another, with emplacement of dark band material between the plates occurring in response to these motions. The very densely fractured region in the centre of the annulus has not been seen yet at high resolution, so the cause of the dense fracturing and the fate of the original surface materials cannot be determined. Localized heating similar to that proposed for the Conamara Chaos region⁶ is a likely possibility. We conclude that creation of dark bands during plate separation is a result of regional surface stresses opening pre-existing fractures which sub-surface materials exploit incrementally. $\square$

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Transport properties governed by surface barriers in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$

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One of the most common investigation techniques of type-II superconductors is the transport measurement, in which an electrical current is applied to a sample and the corresponding resistance is measured as a function of temperature and magnetic field. At temperatures well below the critical temperature, T_c , the resistance of a superconductor is usually immeasurably low. But at elevated temperatures and fields, in the so-called vortex liquid phase, a substantial linear resistance is observed¹. In this dissipative state, which in anisotropic high-temperature superconductors like $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ may occupy most of the mixed-state phase diagram, the transport current is usually assumed to flow uniformly across the sample as in a normal metal. To test this assumption, we have devised a measurement approach which allows determination of the flow pattern of the transport current across the sample. The surprising result is that, in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ crystals, most of the current flows at the edges of the sample rather than in the bulk, even in the highly resistive state, due to the presence of strong surface barriers. This finding has significant implications for the interpretation of existing resistivity data and may be of importance for the development of high-temperature superconducting wires and tapes.

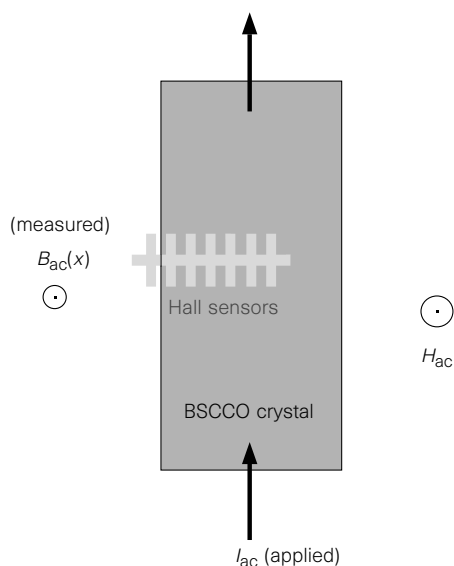


Figure 1 Experimental set-up. A schematic top view of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ crystal ($1.5 \times 0.15 \times 0.01$ mm) attached to an array of seven GaAs/AlGaAs two-dimensional electron-gas Hall sensors underneath the sample. The sensors have an active area of $10 \times 10 \mu\text{m}^2$ and are $10 \mu\text{m}$ apart. There is one sensor outside the sample; the other six span more than half of the sample width. Magnetic field H_{ac} is applied perpendicular to the sample surface. An a.c. current I_{ac} is applied to the crystal through electrical contacts. The sensors are used to probe the perpendicular component of the self-induced a.c. field, B_{sc} , generated by the a.c. current.

The experiments were carried out on several $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (BSCCO) crystals ($T_c \approx 88\text{ K}$) with typical dimensions of $1.5 \times 0.15 \times 0.01\text{ mm}$. The crystals were attached directly to the surface of a linear Hall-sensor array consisting of seven $10 \times 10\text{ }\mu\text{m}$ sensors as shown schematically in Fig. 1. A d.c. magnetic field, H_{dc} , was applied perpendicular to the surface (parallel to the c -axis of the crystal). A low-frequency (65–400 Hz) a.c. current, I_{ac} , in the range 0.4–10 mA (r.m.s.), was applied to the sample using gold contacts. The findings described below are independent of the frequency and the amplitude of I_{ac} , and so only data obtained for 4 and 10 mA at 72.8 Hz are presented. In this study, we have used a sensitive a.c. technique to measure the field profile $B_{ac}(x)$ across the sample, where $B_{ac}(x)$ is the self-induced field of the transport current I_{ac} . This method differs from the measurements of $B_{dc}(x)$ induced by H_{dc} which provide valuable information about the local magnetization or susceptibility of the sample^{2,3}. The present study requires high resolution because B_{ac} is of the order of 0.1 G as compared to the typical B_{dc} of 1,000 G. The use of the a.c. mode provides the necessary separation between the field B_{ac} due to transport current, and the field B_{dc} due to the applied field. The transport-current distribution across the sample is then determined from the experimental $B_{ac}(x)$ profiles.

In a highly dissipative state, the current is expected to flow uniformly across the sample as in a normal conductor, as shown on the left in Fig. 2a. In this case, the resulting vertical component of the self-induced field is given by the solid curve on the right in Fig. 2a, as calculated using the Biot–Savart law⁴. At low temperatures, on the other hand, material disorder pins the vortices and prevents their motion, resulting in a finite critical current. In this case the transport current is expected to flow in a way similar to the

case of the Meissner state where $B_{ac}(x)$ is expelled from the sample^{5,6} as shown in Fig. 2c. As the superconductor is cooled down in the presence of H_{dc} , a monotonic increase of the critical current is expected and therefore a gradual transition from B_{ac} of Fig. 2a to Fig. 2c should occur^{5,6}.

Yet there is another important mechanism, the Bean–Livingston surface barrier⁷, which is usually not taken into account in transport studies. Here we use the term surface barrier to refer to both the Bean–Livingston and a similar geometrical barrier mechanism⁸. Because the presented data are mainly for $H_{dc} > H_{c1}(T)$ (the lower critical field), the contribution of the geometrical barrier is negligible in this case. In order to enter or leave a superconductor, a vortex has to overcome a potential barrier at the surface. This barrier is the result of competition between two forces acting on the vortex near a parallel surface: an inward force due to the presence of the shielding currents, and an outward force due to the attraction between the vortex and its fictitious mirror image outside the sample. In the presence of significant surface barrier, the transport current flows at the edges of the sample, where the vortices enter and leave the superconductor, in order to drive the vortices over the barrier. $B_{ac}(x)$, calculated assuming that the entire current flows at the edges, is shown by the solid curve in Fig. 2b. This field profile is quite different from that of the uniform flow in Fig. 2a. In particular, the signs of $B_{ac}(x)$ within the sample are opposite.

It has been shown that surface barriers have an important contribution to the hysteretic magnetization of clean high- T_c superconductors^{9–11}. These barriers govern the magnetization below the so-called irreversibility line. Owing to practical sensitivity limitations, transport measurements are usually carried out above this irreversibility line where the magnetization is reversible and

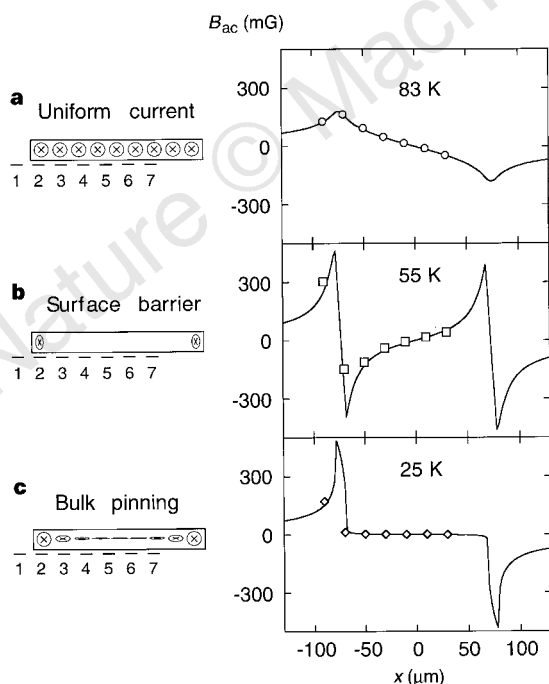


Figure 2 Schematic cross-section of the sample with the attached Hall sensors (left) and the corresponding field profiles $B_{ac}(x)$ at three temperatures (right). Open symbols are the self-field measured by the sensors ($H_{dc} = 1,000\text{ G}$, $I_{ac} = 4\text{ mA}$), and the solid lines are the calculated field profiles for 4 mA current using the Biot–Savart law. **a**, At elevated temperatures, the current flows uniformly across the sample. **b**, At intermediate temperatures practically all the current flows at the edges of the superconductor owing to surface barriers. In this case the field inside the sample has an inverted profile relative to the uniform-current-flow case. **c**, At low temperatures, strong bulk pinning is present which results in zero B_{ac} within the sample.

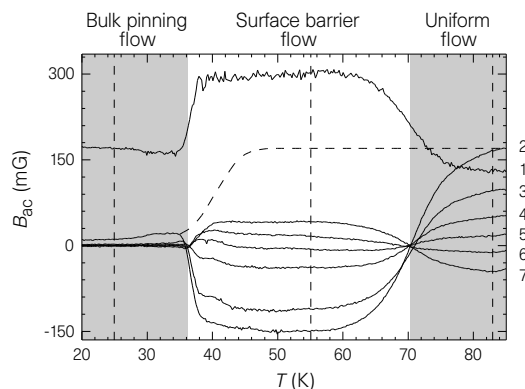


Figure 3 Self-induced field $B_{ac}(x)$ generated by 4 mA a.c. current as a function of the temperature during cooling of the $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ crystal in a field $H_{dc} = 0.1\text{ T}$. The curves are labelled according to the sensor numbers as indicated in Fig. 2. The vertical dashed lines mark the temperatures for which the B_{ac} values are plotted as a function of the sensor location in Fig. 2. At elevated temperatures the current flows rather uniformly across the sample (83 K profile in Fig. 2a). Surface barriers dominate the vortex dynamics over a wide range of intermediate temperatures where the currents flow at the edges of the sample (55 K profile in Fig. 2b). At the crossing point at 70 K, half of the current flows in the bulk and half at the edges causing cancellation of the field in the central part of the sample (superposition of profiles **a** and **b** of Fig. 2). At low temperatures, strong bulk pinning prevents vortex motion, resulting in zero B_{ac} in the sample. The dashed curve indicates the expected field at sensor 2 if the current flow were governed by the bulk vortex properties as commonly assumed. The observed inversion of the field profile is caused by the dominant role of surface barriers.

hence surface effects are expected to be of no importance. The effect of surface barriers on transport measurements has been considered theoretically¹², and some studies have suggested that surface barriers may be of significance¹³, in particular as a source of voltage noise^{14,15}. The flow patterns of the transport current have been tested in several investigations of thin films and tapes^{16–19} but these studies have focused on the critical state behaviour and sample inhomogeneities rather than on surface barriers.

Figure 3 shows the self-field B_{ac} as measured by the sensors on cooling the BSCCO crystal in $H_{dc} = 0.1$ T and $I_{ac} = 4$ mA. The curves are labelled by the sensor number as indicated in the left-hand side of Fig. 2. At elevated temperatures, $B_{ac}(x)$ decreases monotonically across the sample (sensors 2–7) owing to a uniform transport current. The measured field profile at 83 K is shown in Fig. 2a (right) by the open symbols, along with the calculated field profile for 4 mA uniform current shown by the solid curve. The fit clearly indicates that at high temperatures the transport current flows uniformly across the width of the crystal. At low temperatures, $T < 36$ K in Fig. 3, $B_{ac}(x)$ is finite only outside the bulk of the sample (sensor 1, and sensor 2 which is close to the edge) and is zero inside the sample. This behaviour indicates strong bulk pinning and a finite critical current. Figure 2c (right) shows the measured field profile at 25 K and the corresponding calculated curve for this case. At intermediate temperatures, if the surface barrier is neglected, one expects a gradual crossover from a uniform current flow to bulk pinning as indicated by the dashed line for sensor 2 in Fig. 3. However, the measured B_{ac} is drastically different and displays a negative signal which cannot be explained by any model based on bulk vortex properties. This inversion of the polarity of B_{ac} at $T = 70$ K for all sensors within the sample is the result of current flow at the edges of the sample due to the presence of surface barriers. The measured field profile at $T = 55$ K is shown in Fig. 2b (right). The solid curve is calculated for the 4 mA current flowing entirely at the edges of the sample. The clear agreement with the data indicates that the surface barrier completely dominates the vortex dynamics, and as a result, practically the entire current, within experimental resolution of few per cent, flows at the edges of the

sample. Figure 3 shows that at high temperatures the current flows uniformly, then crosses over to a complete surface flow over a wide range of temperatures, and finally changes to bulk-pinning-like flow only at low temperatures.

The above results have important implications for the interpretation of transport measurements. Figure 4 shows the resistance transition in the same BSCCO crystal at various applied fields. Such measurements are usually interpreted in terms of bulk resistivity caused by, for example, viscous vortex flow or pinning. Apparently, only the very top region of our data, where the current flows uniformly in the bulk (dark lines in Fig. 4), reflects bulk vortex properties. Most of the data shown by the grey lines, however, do not reflect bulk vortex properties but rather the properties of the surface barrier. The situation can be roughly represented by the two equivalent circuits at the top of Fig. 4. At elevated temperatures, the sample can be regarded as a uniform resistor. But at lower temperatures (grey lines) the surface barriers act as low resistance shunts, and hence practically all the current flows at the edges. As a result, the total measured resistance is that of the surface barrier rather than that of the bulk.

An alternative way of describing the process is by noting that the measured resistance always reflects the flow rate of the vortices across the sample. However, the flow rate, rather than being determined by bulk vortex pinning and viscous drag, is determined by the hopping rate over the surface barrier¹². This activation is the rate-limiting process impeding vortex motion. Vortex flow rate over the barrier has to be equal to that in the bulk. As a result practically all of the current flows at the edges in order to provide the large force required to overcome the barrier, while only a very small fraction of the current flows in the bulk in order to overcome the small bulk viscous drag. The apparent measured resistance is thus orders of magnitude lower than the true bulk resistance. In other words, the vortices in the bulk are much more mobile, and the corresponding viscous drag coefficient and the pinning force are much smaller, than what is usually deduced from transport measurements^{20–23}.

In addition, resistance of BSCCO crystals commonly displays thermally activated Arrhenius behaviour. In view of the work reported here, such Arrhenius behaviour is caused by the thermal activation over the surface barrier¹² and does not reflect the bulk pinning, as is usually interpreted^{20,21}. Another interesting observation is the recently reported sharp drop in resistance at the first-order vortex lattice melting transition in BSCCO^{23–25}, which is seen, for example, in the 300 Oe data in Fig. 4 at about 65 K. Such a drop is usually ascribed to the sharp onset of bulk pinning on solidification of the lattice^{26,27}. Analysis of the temperature dependence of the current distribution at 300 Oe indicates that even this feature mainly reflects a sharp change in the surface rather than bulk properties, as most of the current still flows at the edges of the crystal and the vortices are unpinned both above and below the melting transition. This does not mean that the bulk properties do not change, but rather that the transport measurements do not probe the true bulk vortex dynamics at the transition. Finally, several magneto-optical investigations have shown that, in BSCCO tapes and wires, the current distribution often peaks at the interface between the superconducting filaments and the Ag sheath rather than within the bulk of the filaments¹⁹. It is thus possible that the surface barriers are also of importance to the current-carrying capability of the commercial wires in a way similar to the behaviour of the single crystals. □

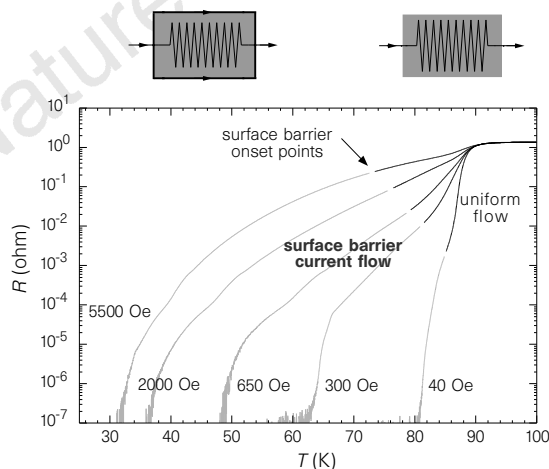


Figure 4 Resistance of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ crystal as a function of temperature at the indicated applied fields ($I_{ac} = 10$ mA). Only in the region of the black curves does the current flow uniformly across the sample (at least 90% of the current). In the region where the curves are grey most of the current flows at the edges of the sample owing to strong surface barriers. The top left diagram illustrates how the surface barriers shunt the current flow in a large part of the phase diagram (surface barrier flow region in Fig. 3), and as a result the measured resistance may be orders of magnitude lower than the true bulk resistance of the superconductor. The top right diagram illustrates the uniform flow case where the surface barriers are unimportant.

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Icosahedral packing of B_{12} icosahedra in boron suboxide (B_6O)

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Objects with icosahedral symmetry (I_h) bear a special fascination; natural examples are rare, but include radiolaria¹ and virus particles (virions)². The discovery³ of C_{60} , a molecule in the shape of a truncated icosahedron with I_h symmetry, has aroused widespread interest. In 1962, Mackay⁴ described a radiating packing of spheres in I_h symmetry, in which the centres of successive shells of spheres lie on the surfaces of icosahedra. There has been extensive investigation of the conditions under which such packing might be realized in assemblies of atoms or of

molecules such as C_{60} (ref. 5). Here we report the preparation, at high temperatures and pressures, of boron suboxide (B_6O) in which the preferred form of the material is as macroscopic, near-perfect, regular icosahedra, similar to the multiply-twinned particles observed in some cubic materials. A major difference is that B_6O has a rhombohedral structure that nearly exactly fits the geometrical requirements needed to obtain icosahedral twins. These icosahedral particles have a structure that can be described as a Mackay packing of icosahedral B_{12} units, and thus has long-ranged order without translational symmetry.

The icosahedron (regular polyhedron with 20 triangular faces) is a forbidden morphology for single crystals: because of the absence of 5-fold axes in classical crystallography, the external forms of single crystals cannot exhibit such symmetry. However, some 'crystalline' objects without three-dimensional periodicity do not comply with this rule: in chrysotile and polygonal serpentine minerals, the fibre axis has 5-fold symmetry^{6,7}. Five-fold cyclic twins, usually imperfect, have long been known in minerals such as cassiterite (SnO_2), pentagonite⁸ or diamond^{9,10}. Small multiply-twinned particles (MTPs) are known with the form of pentagonal bipyramids (decahedra), and also, rarely, as icosahedra of elemental metals^{11–13} and of chemical-vapour-deposited (CVD) diamond¹⁴. Many quasicrystals have icosahedral space-group symmetry and, in some cases, crystallize in pentagonal dodecahedra¹⁵ and rhombic triacontahedra¹⁶ with icosahedral symmetry.

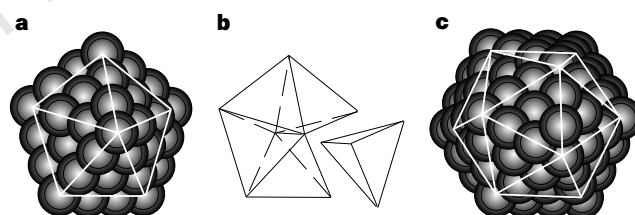


Figure 1 **a**, An element of decagonal sphere packing. **b**, A decagon (pentagonal bipyramid) with one of its five component tetrahedra removed. **c**, An element of icosahedral (Mackay) sphere packing. The tetrahedra in c.c.p. metals are derived from the familiar 'pile-of-cannonballs' densest packing of atoms with density ρ_{cp} . The density of the decagonal sphere packing²¹ is a maximum if the tetrahedra are distorted so that the length of the edge common to the five tetrahedra is increased by a factor of $\sqrt{3}\sin(\pi/5) = 1.018$. The packing density is then $0.991\rho_{\text{cp}}$ (**a**). Thus only a small strain is required to produce decahedral multiply-twinned particles (MTPs) from cubic parent material^{9,11,12}. Icosahedral MTPs consist of 20 tetrahedra meeting at a vertex; the tetrahedra must be more distorted from ideality, as described in the text. The density of the sphere packing is now $5/(2\sqrt{5} + \sqrt{3})\rho_{\text{cp}} = 0.929\rho_{\text{cp}}$ (ref. 4).

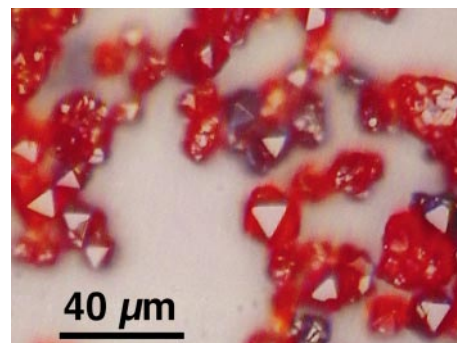


Figure 2 Reflected-light optical image of B_6O icosahedral particles. Triangular faces of individual icosahedra are evident. Individual particles occur as isolated or clustered icosahedra, although rounded particles of very fine-grained sintered material also exist. The colour is consistent with a bandgap around 2 eV (ref. 32).

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Decahedral MTPs of cubic close-packed (c.c.p.) metals such as gold consist of five slightly distorted tetrahedra (Fig. 1a, b). The dihedral angle at the edge common to the five tetrahedra is 72° rather than the 70.5° for regular tetrahedra. Icosahedral MTPs (see Fig. 3b) consist of 20 tetrahedra even more distorted from ideality: the three edges of the outer faces are slightly larger than the three meeting at the centre of the icosahedron¹⁷ by a factor of $\sqrt{(8/(5 + \sqrt{5}))} = 1.051$. The solid angle at the centre equals $4\pi/20$ sr instead of $3 \cos^{-1}(1/3) - \pi = 4\pi/22.8$ sr for a regular tetrahedron. It follows that to avoid strain, individual crystals in an icosahedral MTP must have a rhombohedral unit cell with $\alpha = \cos^{-1}(1/\sqrt{5}) = 63.44^\circ$ (the angle between adjacent 5-fold axes of an icosahedron) equivalent to $c/a = (\sqrt{3}/4)(3 + \sqrt{5}) = 2.267$ in hexagonal setting. This difference from ideal c.c.p. ($\alpha = 60^\circ$, $c/a = \sqrt{6} = 2.45$) explains why icosahedral MTPs are rare in c.c.p. metals and occur only as nanoparticles or as particles grown under conditions far from equilibrium (CVD diamond).

Boron suboxide (nominally B_6O) belongs to a family of α -rhombohedral boron-rich compounds^{18,19}, that combine useful properties of high hardness, low density and chemical inertness. In the course of high-pressure, high-temperature (HP-HT) syntheses of B_6O , we maintained mixtures of B and B_2O_3 at $1,700^\circ\text{C}$ for 30 minutes between 4 and 5.5 GPa using a multi-anvil press^{20,21}. Orange-red grains up to $30\ \mu\text{m}$ in diameter, with almost perfect icosahedral morphology, formed in a soft, water-soluble matrix of boron oxides (Figs 2, 3). Analysis by parallel electron energy-loss spectroscopy (PEELS) showed a stoichiometry $B_6O_{0.96 \pm 0.08}$, closer to the nominal formula than previous material synthesized at lower pressures^{22–24}.

Powder X-ray diffraction of B_6O samples with icosahedral morphology displays the expected rhombohedral structure^{23–25}. Scanning electron microscopy (SEM) reveals near perfect icosahedra (Fig. 3a) or intergrown icosahedra. Some icosahedra have well developed re-entrant angles at the apices, indicative of twinning (Fig. 3c). Transmission electron microscopy (TEM) images show icosahedral particles as small as 20 nm, with bright- and dark-field contrasts confirming that they are MTPs. Euhedral particles under 100 nm in diameter are also occasionally observed by TEM associated with the icosahedra. High-resolution TEM images of the smallest icosahedra along the 5-fold axis unambiguously show the twinning (Fig. 3d). Although stacking faults occur near many twin planes, the twin individuals have a low density of defects, which indicates minimal strains. Similar observations can be made on crushed icosahedra of larger dimensions. In comparison, diamond MTPs display extensive twinning¹⁰. Selected-area electron diffraction patterns of properly oriented single icosahedra of B_6O (Fig. 3e) beautifully illustrate the 5-fold symmetry and the near perfection of the icosahedral twinning.

The α -rhombohedral (space group $R\bar{3}m$) boron structure²⁶ is an approximate close packing of icosahedral B_{12} units with $c/a = 2.55$. The same packing of B_{12} units occurs in B_6O (ref. 27) in which O atoms are inserted between the icosahedra (Fig. 4a), decreasing the c/a ratio. In B_6O_{1-x} , the axial ratio decreases with increasing O content. The unit cell parameters for one of our samples were refined from powder X-ray diffraction data to $a = 5.3902(1)\ \text{\AA}$, $c = 12.3125(2)\ \text{\AA}$, which gives a c/a ratio of 2.284 ($\alpha = 63.1^\circ$), within 1% of the value required for unstrained icosahedral MTPs.

In the case of β -rhombohedral boron^{26,28}, the most stable allotrope, a fragment of the structure can be described by $B_{12}(B_{12})_{12}$

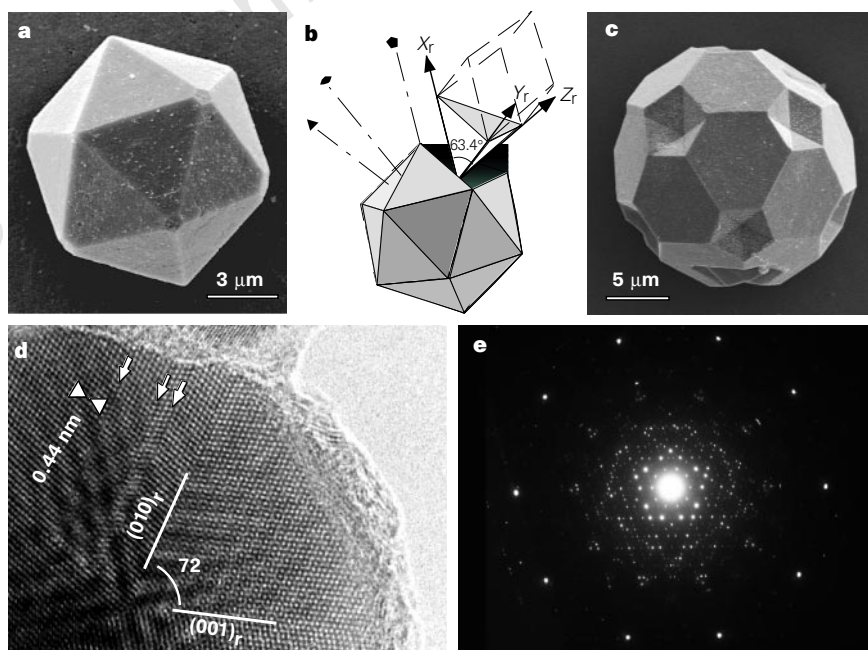


Figure 3 Morphology of B_6O MTPs. **a**, Scanning electron microscopy (SEM) image of an isolated icosahedron. **b**, Descriptive model of a regular icosahedron consisting of 20 tetrahedral individuals, illustrating the geometric relationship between a tetrahedral individual and rhombohedral cell. Icosahedral MTPs are described in the rhombohedral system (denoted by r) by individuals twinned along the $\{100\}_r$ faces, with common $\{100\}_r$ edges, and with the $[111]_r$ directions (c hexagonal) coinciding with the 3-fold axes passing through the centres of the icosahedron and the triangular faces. The rhombohedral unit cell of B_6O with $\alpha = 63.1^\circ$ closely matches the one for ideal icosahedral twinning, $\alpha = 63.4^\circ$. The symmetry elements of the icosahedron are also shown. **c**, SEM image of B_6O MTPs with well-developed faces forming re-entrant angles at the apices, indica-

tive of twinning. This grain has the same morphology as the C_{60} molecule. **d**, High-resolution TEM image of a portion of a B_6O MTP in $[100]_r$ zone-axis orientation (that is, seen along the 5-fold axis of the icosahedron). Additional 'stacking faults' (arrowed) are visible parallel to one of the twin planes. The moiré effect visible in the central part of the particle results from the superposition of the other twin individuals. (The 'stacking faults' shown here in fact correspond to 'sandwiches' or lamellae of twinned material between two stacking faults.) **e**, Selected-area electron diffraction pattern of a B_6O icosahedral MTP, showing the 5-fold symmetry. The intense spots defining the inner pentagonal pattern lie at $1/0.436\ \text{nm}^{-1}$ ($1/d(100)_r$) and $1/0.37\ \text{nm}^{-1}$ ($1/d(110)_r$).

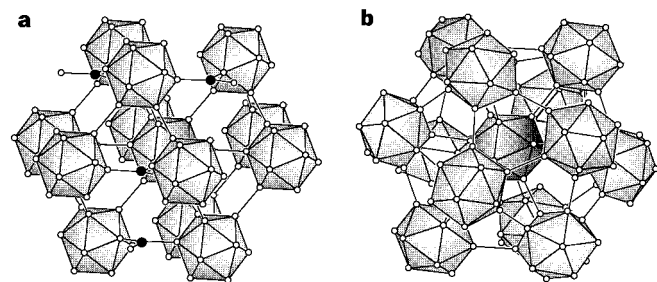


Figure 4 **a**, A part of the B_6O structure showing c.c.p. packing (slightly distorted) of B_{12} icosahedra with O atoms (filled circles) in the close-packed layers. Each O is bonded to a B in three separate icosahedra. The centres of the outer 12 icosahedra are at the vertices of a cuboctahedron. **b**, A fragment of the β -rhombohedral boron structure. The central B_{12} unit is surrounded by 12 other B_{12} icosahedra whose centres are at the vertices of an icosahedron. The whole assembly is an icosahedron of icosahedra ('super-icosahedron'). Such a super-icosahedron also occurs in YB_{66} (ref. 27).

structural units: a central B_{12} icosahedron is surrounded icosahedrally by 12 other B_{12} units forming a 'super-icosahedron' (Fig. 4b). In our icosahedral MTPs of B_6O , such a super-icosahedron may serve as the nucleus for the observed icosahedral packing of B_{12} units. Growth occurs by addition of successive layers of B_{12} icosahedra to each triangular face. The realization of the icosahedral Mackay packing⁴ in B_6O and its macroscopic manifestation as icosahedral MTPs results from a combination of circumstances; these include the stability of the super-icosahedral unit that can serve as a nucleus and the appropriate rhombohedral distortion from ideal c.c.p. allowing unstrained icosahedral twinning. This distortion is closely related to the oxygen content, which is maximized in our high-pressure experiments²⁰.

It is remarkable that the bonding between the B_{12} units is consistent with the Mackay packing, similar to that encountered in β -rhombohedral boron in the centre, and to that of α -rhombohedral boron within the twin individuals. The twin boundaries and stacking faults observed near the twin boundaries correspond locally to a hexagonally close-packed type of stacking of the B_{12} icosahedra, rather than the normal (distorted) c.c.p. of α -rhombohedral boron. Such planar defects are expected to be of low energy because they do not affect the bonding with the first-neighbouring B_{12} units.

The B_6O MTPs are surely among the largest of self-assembled regular icosahedral particles found so far: there are $\sim 10^{14}$ atoms in a 20- μm icosahedron compared with 10^7 for typical virus particles. An additional feature of interest is that boron suboxide is known to be extremely hard²⁹, with abrasion qualities similar to those of diamond³⁰. The icosahedral morphology of the grains makes them potentially useful for high-wear-resistance applications. Despite the uniaxial (trigonal) unit cell symmetry, all external faces of the MTPs have the same crystallographic orientation ($\{111\}_T$), and hence surface energy, which is presumably at a minimum. The faces should therefore wear at the same rate. The external icosahedral form, preferred under these high-pressure synthesis conditions, can be seen as a special state of condensed matter with long-range order that is neither three-dimensionally periodic nor quasicrystalline, but nevertheless possessing a higher degree of organization (and higher symmetry) than the individual crystals constituting the MTPs. □

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The timing of Pleistocene glaciations from a simple multiple-state climate model

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The Earth's climate over the past million years has been characterized by a succession of cold and warm periods, known as glacial–interglacial cycles, with periodicities corresponding to those of the Earth's main orbital parameters; precession (23 kyr), obliquity (41 kyr) and eccentricity (100 kyr). The astronomical theory of climate, in which the orbital variations are taken to drive the climate changes, has been very successful in explaining many features of the palaeoclimate records¹. Nevertheless, the timing of the main glacial and interglacial periods remains puzzling in many respects^{2–5}. In particular, the main glacial–interglacial switches occur approximately every 100 kyr,

but the changes in insolation forcing are very small in this frequency band. Similarly, an especially warm interglacial episode, about 400,000 years ago⁷, occurred at a time when insolation variations were minimal. Here I propose that multiple equilibria in the climate system can provide a resolution of these problems within the framework of astronomical theory. I present two simple models that successfully simulate each glacial–interglacial cycle over the late Pleistocene epoch at the correct time and with approximately the correct amplitude. Moreover, in a simulation over the past 2 million years, the onset of the observed prominent ~100-kyr cycles around 0.8 to 1 million years ago is correctly reproduced.

Although the 23-kyr and 41-kyr periodicities found in the palaeoclimate records seem to be almost linearly related to the insolation forcing², the largest climate variations of the past million years occur approximately every 100 kyr though the corresponding eccentricity changes are far too small to force the changes. Many hypotheses have been formulated to explain this ‘100 kyr problem’, sometimes even involving other astronomical parameters³. Most of the suggested mechanisms are based on a nonlinear response of the ice-sheet dynamics to the forcing⁴ or internal oscillations of the climate system^{5,6}. Although some of these models compare well with the geological record in the spectral domain, all of them fail to reproduce the correct amplitude and phase of each glacial–interglacial cycle. In particular, one of the most prominent interglacial events, isotope stage 11, occurs at a time when the insolation variations are the smallest (around 400 kyr before present, BP) and therefore poses a strong challenge^{4,7}.

A multiple-state representation of the climate system may help solve these problems. The 100-kyr climate signal is not linearly related to the forcing⁸, and thresholds between different states provide the simplest possible nonlinear mechanism. Furthermore, multiple states are known to exist in the climate system: in particular, there is increasing evidence that the ocean is such a system. The threshold model presented here is actually derived from a multiple-state ocean box-model⁹.

The glacial–interglacial cycles are closely associated with the build-up and melting of the Laurentide and the Fennoscandian ice sheets at high northern latitudes. It is generally assumed, as a crude approximation, that summer insolation at high northern latitudes controls the ice-sheet volume, and hence the global climate. This is supported by the strong correlation^{2,8} between global palaeoclimate records and the summer insolation at high northern latitudes. Therefore, this parameter is used in simplified models as the main external forcing. In the following, the term ‘insolation’ will refer to the daily insolation at 65° N at the summer solstice as computed by Berger¹⁰.

I present two simple models that reproduce reasonably well the succession of glacial–interglacial cycles over the late Pleistocene. Whereas the power spectrum of the geological record is dominated by the 100-kyr periodicity over about the past 1 Myr, the 41-kyr periodicity seems to be dominant before¹¹. I will therefore concentrate on the latest part of the record. The first model has three distinct states called **i** (interglacial), **g** (mild glacial) and **G** (full glacial). The second model is an extension of the first one.

Stommel¹² was the first to show that the oceanic thermohaline circulation could have multiple steady states. This was further verified in more sophisticated models^{13–16}. The idea of a three-state structure came directly from the study of a simplified ocean model⁹ whose dynamical structure is used here as our basic assumption.

If it is assumed that, depending on the insolation forcing and the ice volume, the climate system can enter three different regimes, and that the transitions occur according to Fig. 1, then a very simple model with few tunable parameters is obtained. This model undergoes an **i–g** transition as soon as the insolation falls below i_0 . A **g–G** transition happens when the ice volume exceeds a threshold $v_{\max}$,

and finally a transition **G–i** occurs when the insolation increases above i_1 . These transitions are assumed to be one-way: a **g–i** transition is impossible because the required insolation threshold is assumed to be beyond reach. The **g** regime is located between the **i** and **G** ones when, and only when, the ice volume is low: it should be low at the end of the **i** regime, and high after the **G** regime. Therefore, the **G–g** and **i–G** transitions are forbidden.

Without any explicit ice volume in this first model, I assume that the ice sheet needs some minimal time t_g in order to grow and exceed the volume $v_{\max}$ (**g** regime duration larger than t_g) and that the insolation maxima preceding the **g–G** transition must remain below the level i_3 . The **g–G** transition then can occur at the next insolation decrease, when it falls below i_2 .

Starting this conceptual model at the end of isotope stage 22 in a **G** regime, in an insolation minimum (at 875 kyr), we obtain the succession of states shown in Fig. 2. All the nine succeeding climate **G–i** transitions correspond to the main isotope transitions seen in the geological record. In particular, isotope stage 11.3 is correctly predicted at ~400 kyr BP. This model furthermore explains why stage 7.5 is warmer than the succeeding stage 7.3, although both insolation and ice volume would tend to suggest the opposite^{1,17}: only stage 7.5 is a ‘true’ interglacial, while 7.3 corresponds to a particularly ‘mild’ glacial. The anomalous duration^{1,7} of stage 11.3 is similarly explained by the particular shape of the forcing at this time.

This model is not very sensitive to parameter changes. Different threshold values will slightly offset the transitions by a few hundred years, but the overall shape will remain the same for a broad range of values. There is no significant changes when i_0 is between –0.97 and –0.64, i_1 between –0.23 and 0.32, i_2 between –0.30 and 0.13, i_3 between 0.97 and 1.16, and t_g between 27 kyr and 60 kyr. Even when the parameters are out of these bounds, the changes are minor: when i_0 is between –0.63 and –0.09, the succession of regimes remains the same except for present time, which becomes a **g** regime. When i_1 is chosen between 0.33 and 0.87, only the duration of stage 11.3 changes to become more comparable to other interglacial stages. The model is not very sensitive to the initial state: starting at 875 kyr BP in the wrong regime (**g** or **i**), the next two transitions are eventually misplaced, but the model rapidly recovers and results are unchanged afterwards (between 700 kyr and present).

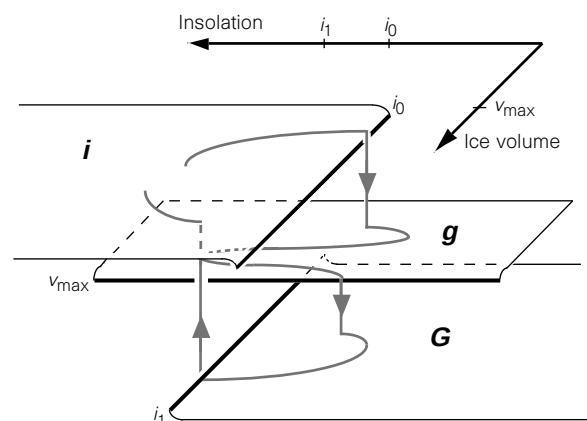


Figure 1 Schematic structure of the multiple-state system used. The climate is assumed to have three distinct regimes; **i**, **g** and **G**. The **i–g** transition is triggered when the insolation falls below the threshold i_0 , the **g–G** transition occurs when the ice volume exceeds $v_{\max}$ and the **G–i** transition occurs when the insolation increases above the level i_1 . Without any explicit ice volume in the first model, the $v_{\max}$ threshold is replaced by a minimal duration t_g for the **g** regime and the requirement that the previous insolation maximum is below a level i_3 : the **g–G** transition occurs then at the next insolation decrease (below i_2). In the second model, the ice volume is explicitly represented.

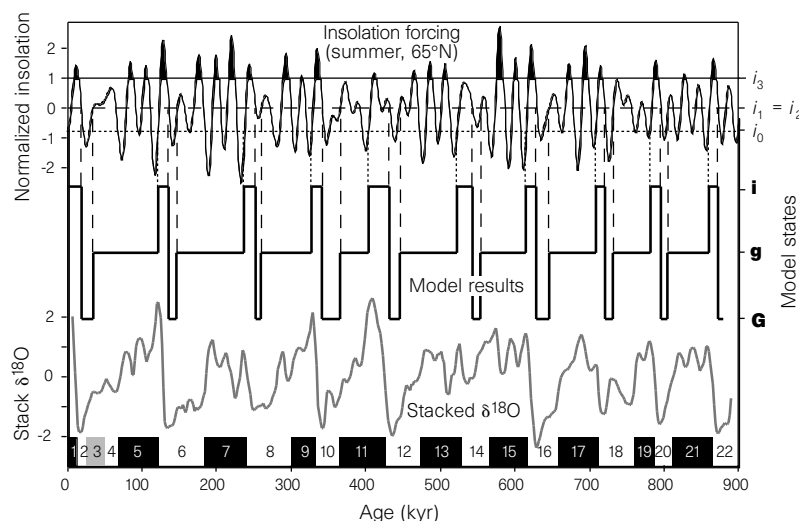


Figure 2 Results from the first idealized model (middle curve). The transitions (dashed vertical lines) are triggered by threshold (dashed horizontal lines) crossings of the insolation (upper curve, normalized to zero mean and unit variance). Insolation maxima above i_3 are in black: full glacial regimes **G** are associated with maxima below i_3 . A stack of several geological oxygen isotope records²⁴ is plotted below for comparison, as well as the conventional isotope stage numbers. The threshold values are, in variance units, $i_0 = -0.75$, $i_1 = i_2 = 0$, $i_3 = 1$. The minimal duration of a **g** regime is $t_g = 33$ kyr.

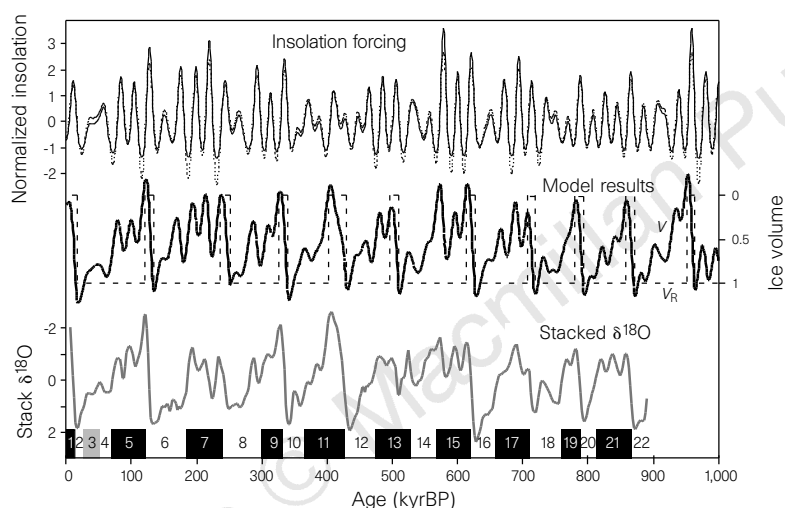


Figure 3 Results from the second model (thick middle curve). The value v_R is also plotted (dashed middle curve) in order to differentiate between **i** regimes ($v_R = 0$) and **g** or **G** regimes ($v_R = 1$). The model enters a **G** regime when the ice volume exceeds the value $v_{\max} = 1$. The forcing F used here (continuous line) is a 'smoothed truncation' of the insolation used before (dashed line). An oxygen isotope stack record²⁴ is plotted below for comparison. The thresholds are, in variance units, $i_0 = -0.75$, $i_1 = 0$. The time constants are $\tau_i = 10$ kyr, $\tau_G = \tau_g = 50$ kyr and $\tau_F = 25$ kyr.

The same results can be obtained using another insolation curve (north of 35°N, from mid-April to mid-August, or with the Laskar¹⁸ insolation curves) with only minor adjustments in the parameters.

These results highlight the fact that the interglacial stages defined in the isotope records are not directly associated with the largest maxima in the insolation curve. On the contrary, they systematically occur after the small ones¹⁹. Indeed, these small maxima eventually trigger a full glacial stage, then followed by an interglacial.

To go one step further, I constructed a differential version of the previous model. This second model still has the three regimes **i**, **g** and **G**, but the ice volume is allowed to change continuously. The criteria for the **i**–**g** and the **G**–**i** transitions are the same as before with the same threshold values. The **g**–**G** transition now occurs when the ice volume exceeds the value $v_{\max}$ without any condition on the insolation. The model is, except for the thresholds, a simple linear one:

$$\frac{dv}{dt} = (v_R - v)/\tau_R - F/\tau_F$$

where v is the ice volume, R is the current climate regime ($R = \mathbf{i}, \mathbf{g}, \mathbf{G}$), v_R are the reference ice volumes for the different regimes, F is the forcing, τ_R and τ_F are time constants. The ice volume is normalized to unity: $v_g = v_G = v_{\max} = 1$, $v_i = 0$.

The forcing used here is a 'smoothed truncation' of the insolation in the previous model: F is computed using a truncation function

f on the insolation, then normalizing to unit variance and zero mean, with:

$$f(x) = \frac{1}{2} \left(x + \sqrt{4a^2 + x^2} \right)$$

This results in a 'full' truncation: $f(x) = (x + |x|)/2$, when $a = 0$, and no truncation at all (after normalization), when $a = \infty$. We choose $a = 1$. This empirical adjustment accounts for the lower sensitivity of the ice volume during colder periods.

Starting at 1 Myr BP in a **g** regime with an ice volume $v_0 = 0.75$, we obtain results (Fig. 3) in good agreement with the isotope data. In this new experiment, the **i** regime of stage 13 occurs at a different time. Without reliable global estimates of the temperature during this time periods, it is difficult to prefer one or the other solution. No attempt was made to 'tune' the model to obtain a 'best possible' agreement with the data.

This second model is also quite robust. It is particularly insensitive to changes in τ_i and τ_G : any value of τ_G (0 to infinity) is acceptable and τ_i can be chosen between 0 and 15 kyr. The results will be almost unchanged with τ_g between 47 and 57 kyr, and τ_F between 23 and 29 kyr. The truncation parameter a can be chosen between 0.54 and 1.66. This second model also behaves quite correctly outside the limits given here, except for one or two transitions which will then be misplaced. Quite unexpectedly, stage 11 is one of the most robust features of this model. The most sensitive ones are stage 21, 19 and 3, where the ice volume

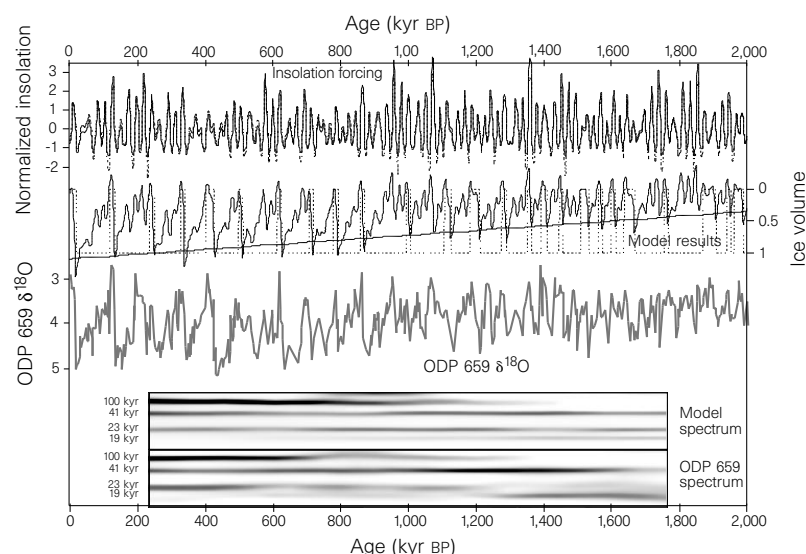


Figure 4 Same as Fig. 3 but with a time-varying threshold $v_{\max}$. The insolation thresholds i_0 and i_1 are the same as before. The time constants are now $\tau_1 = 5$ kyr, $\tau_G = \tau_g = 80$ kyr and $\tau_F = 28$ kyr. The ice volume threshold $v_{\max}$ (oblique thin line) increases linearly from 0.35 to 1.1 and a slight linear trend ($3 \text{ W m}^{-2} \text{ Myr}^{-1}$) is added to the insolation forcing (Laskar solution¹⁸) before truncation. The oxygen isotope record²⁵ from ODP site 659 is used for comparison. An evolutive spectral analysis of the two series is performed using the maximum entropy method and a sliding time window of 500 kyr. In both spectra, the 100-kyr periodicity appears around 1 Myr BP. Many aspects of the two series are quite similar, both in the time and frequency domains.

almost crosses the threshold $v_{\max}$ 'before time' (Fig. 3) and stage 7, where the ice volume barely crosses this threshold. For example, with no truncation on the forcing ($a = \infty$), all transitions remain unchanged except stage 7.3 and stage 3 which become interglacials. Starting in the wrong regime has also an effect limited to the next two cycles, after which the model recovers and results are unchanged. Using different high-latitude summer insolation curves does not affect the results, though the Laskar¹⁸ ones appear preferable. I therefore believe the results from both models to be fairly robust.

In a last experiment covering 2 million years (Fig. 4), the model simulates correctly the onset of 100-kyr glaciations around 0.8–1 Myr BP. Over this time span, the slow tectonic activity induces slight ocean topographic changes and a possible decrease of atmospheric CO_2 concentrations¹⁹. Both will affect the threshold values of our conceptual model. The CO_2 decrease will also directly influence the radiative forcing. The results shown in Fig. 4 are obtained with a linearly increasing $v_{\max}$ threshold value, and a small linear trend added to the radiative forcing. The youngest part of the resulting simulation remains the same as before, and many aspects of the older part also compare reasonably well with the geological record, both in the time and spectral domains. It is nevertheless quite obvious that a more sophisticated model is needed to account for the detailed variability of the climate system over such a period of time.

Multiple states seem to be omnipresent, at least in climate models, and probably in the climate itself. The interpretation of palaeoclimate records of the past million years, in terms of a multiple-state system, seems therefore quite natural. The three-state model presented here seems to be a good candidate to explain some puzzling features of the Quaternary records, in particular the '100 kyr problem' as well as the 'stage 11 problem'. Furthermore, it is quite robust to changes in its parameters. It is not clear which physical components of the climate system may be responsible for such a trimodality, though the ocean is probably the best guess: coupled GCM experiments have shown that the Atlantic thermohaline circulation can be switched on or off with changing freshwater input²⁰ or changing atmospheric CO_2 concentration²¹. Some results on the last glacial inception^{8,22,26} tend also to indicate that ocean circulation changes at high latitude precede the ice-sheet build-up. More generally, high-resolution palaeoclimate records show that abrupt transitions^{23,26} are more the rule than the exception, thus suggesting the existence of thresholds. In any case, and in contrast to recent claims³, this conceptual model clearly demonstrates that the geological record can easily be explained in the framework of the

classical astronomical theory. If multiple states are an important characteristic of the climate system, much effort remains to be made in order to determine the critical thresholds of future climate changes. □

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Energy availability and habitat heterogeneity predict global riverine fish diversity

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Processes governing patterns of richness of riverine fish species at the global level can be modelled using artificial neural network (ANN) procedures. These ANNs are the most recent development in computer-aided identification and are very different from conventional techniques^{1,2}. Here we use the potential of ANNs to deal with some of the persistent fuzzy and nonlinear problems that confound classical statistical methods for species diversity prediction. We show that riverine fish diversity patterns on a global scale can be successfully predicted by geographical patterns in local river conditions. Nonlinear relationships, fitted by ANN methods, adequately describe the data, with up to 93 per cent of the total variation in species richness being explained by our results. These findings highlight the dominant effect of energy availability and habitat heterogeneity on patterns of global fish diversity. Our results reinforce the species-energy theory³ and contrast with those from a recent study on North American mammal species⁴, but, more interestingly, they demonstrate the applicability of ANN methods in ecology.

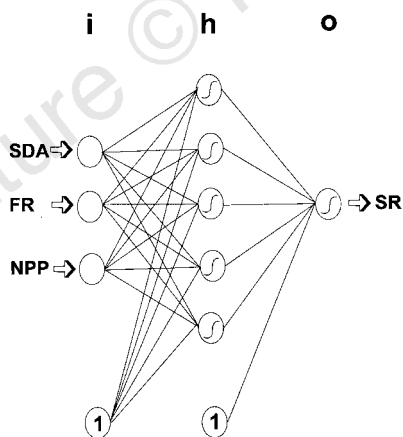


Figure 1 Three-layer feed-forward artificial neural network (ANN) structure used in this work. There are three input neurons (i) for surface of the drainage area (SDA), mean annual flow regime (FR), and net primary productivity (NPP), and only one output neuron (o) which corresponds to fish species richness (SR) in rivers. The hidden layer (h) has five neurons, determined as the optimal configuration that gives lower error during training with minimal computing time. There are two additional bias nodes labelled with a constant input value of 1.0. Initially, the network was trained with a set of 183 rivers and their corresponding parameters for 1,000 iterations. We then examined the capability of the trained network to predict SR with a 'leave-one-out' procedure (see Fig. 4 and statistical analysis).

A central issue in macroecology is to determine the forces that shape large-scale patterns of species richness^{5,6}. Three main hypotheses have been proposed to explain the spatial variability in species diversity. The first, the species-area hypothesis⁷, implies that species richness increases as a power function of surface area; the second, the species-energy hypothesis^{3,8}, predicts that species variation is correlated with energy availability in the system; the third, the historical hypothesis⁹, explains species richness gradients in terms of patterns of recolonization and maturation of ecosystems after glaciation. However, so far none of the three theories has been supported to the exclusion of the other, and many causative factors have been cited even though total available energy has gained currency as a major influencing parameter of species diversity. Here we model processes governing patterns of riverine fish species richness. We use ANNs, known for their capacity to process nonlinear relationships between variables^{1,2}. The data we present are best explained by the hypothesis that both distribution of available energy and habitat heterogeneity limit fish species richness in rivers on a worldwide scale.

Global-scale patterns of fish species richness in rivers have previously been investigated using linear statistical models¹⁰. Results suggest that factors related to components of river size (surface area and flow regime) and energy availability (net primary productivity) are most important in predicting fish diversity, whereas the role of other possible factors (such as contemporary climate and/or history) are of only marginal importance. The effect of contemporary available energy has been demonstrated on different groups of organisms^{10–14}, although some other factors (historical influence, for example) may predict patterns of richness^{9,15}. We have reinvestigated previous work¹⁰ employing ANN methods, which do not require a linear relationship between variables and so may be better suited to model nonlinear phenomena (Fig. 1). ANNs are different from multiple linear regressions in that the relationships between independent parameters and fish species richness (SR) are estimated by an iterative trial-and-error procedure. Each influencing parameter

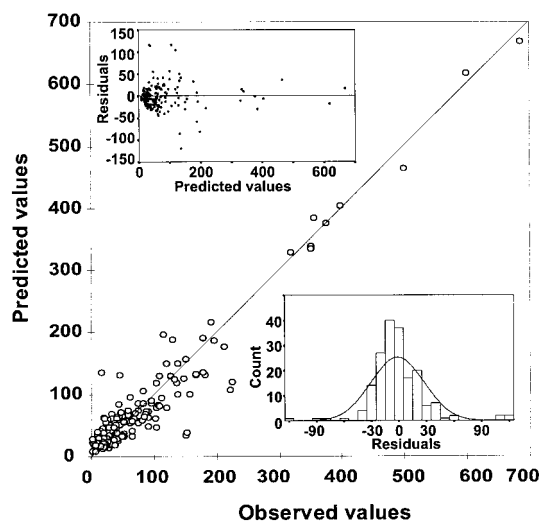


Figure 2 Prediction of fish species richness (SR) using the 3-5-1 artificial neural network (ANN) model shown in Fig. 1. Scatterplots compare predicted and observed SR values. This relationship is highly significant ($n = 183$, $r = 0.958$, $P < 0.0001$). The diagonal line illustrates points at which the predicted value equals the observed value. Top inset, relationship between the residual values obtained from the ANN model and the predicted values. The horizontal line represents points for which residuals equal zero. The relationship shows no obvious sign of dependence of residuals ($n = 183$, $r = 0.018$, $P = 0.805$), which indicates that the ANN model fits the data well. Lower inset, frequency histogram of residuals with most values centred near zero ($n = 183$, mean = -2.00 , s.d., ± 28.82).

is assigned with different weights and the combined weighted values are added to predict SR. We tested two aspects of ANN performance. First, we evaluated the ability of the independent parameters (surface of the drainage area (SDA), flow regime (FR), and net primary productivity (NPP)) to predict SR of new samples, and thus for modelling fish species diversity processes on the global scale. Second, we analysed the contribution profile of each predictor as a measure of sensitivity to match data. For this, we used a three-layer feed-forward (3-5-1) neural network: that is, three input neurons corresponding to the three independent parameters, five hidden neurons determined as the optimal configuration (best compromise between bias and variance) and one output neuron for SR, which was trained using the backpropagation algorithm¹ (Fig. 1). In the past decade, ANN models have been widely applied in different research fields^{1,2} (physics, chemistry, behavioural sciences) but very few studies have focused on the use of ANNs in theoretical ecology and evolution². We now explore this possibility in the context of conservation ecology.

The 3-5-1 ANN model (Fig. 1) accurately predicted the pattern of observed SR on a global scale (Fig. 2). The contribution profiles of the three predictors for explaining SR estimates are illustrated in Fig. 3. The predictive performance of the ANN model gave significant results with 92.9 per cent of rivers achieving a perfect data fit (Fig. 4), thereby increasing the significance of predictions.

Examination of Fig. 3 shows that for FR and NPP parameters, there is a strong positive effect on richness patterns, with a sigmoidal

contribution between the ability of these two variables to match data and SR values. In contrast, the contribution of the SDA variable (that is, river size) contributes little to variation in global fish diversity, with a contribution profile better fitting a gaussian function where the maximum of sensitivity is achieved for median fish richness values and average river sizes (Fig. 3). This conflicts with previous studies showing the importance of drainage surface area on fish species patterns¹⁵⁻¹⁷. The small contribution of surface area as a predictor variable indicates the extent to which previous investigations were strongly influenced by log-linear transformations. SDA and FR could be causally linked and these processes are probably acting together, confounding their effects. However, discharge may be a more direct measure of available habitat diversity because it may implicitly integrate a third dimension in river size, the volume of available water for fish communities^{16,17}.

Interestingly, FR and NPP predictors strongly influence patterns of global-scale SR (Fig. 3). As rivers with high flow regimes may generally contain a greater array of habitat configurations¹⁸, a part of island biogeographic theory⁷, local habitat heterogeneity may induce this increase in global SR. Additionally, the influence of net primary productivity, a measure of energy availability, demonstrates the importance of energy input on riverine fish richness patterns on a worldwide scale. This fish species richness–primary productivity function resembles the logistic model obtained for the three species richness–evapotranspiration relationship¹⁴. Climatic

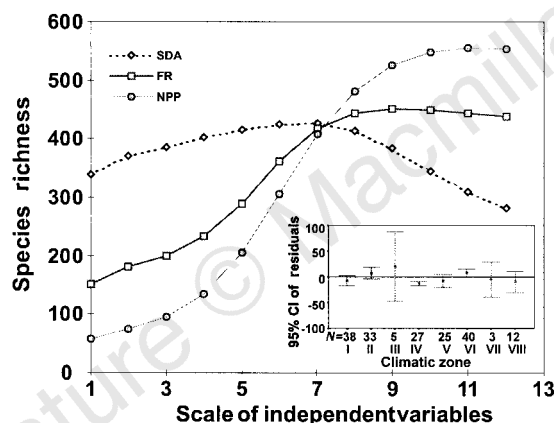


Figure 3 Contribution of the three independent variables (SDA, FR and NPP) used in the 3-5-1 ANN model. Sensitivity profiles explain SR (see Fig. 1 for abbreviations). Contribution of each independent variable to SR estimates is assessed by visual examination of nonlinear profiles: NPP and FR variables have a sigmoid function, with the NPP curve showing a higher range of variation than the FR function; SDA variable better fits a gaussian function. Both NPP (which explained 43% of the total variance) and FR (31%) show a predominant positive effect on SR, whereas SDA (26%) has a weaker effect on SR value. The relative importance of influencing parameters on SR was calculated according to refs 29 and 30. Inset, residual values generated by the ANN model plotted against the 8 categories of climatic zones (I to VIII). Bars represent 95% confidence intervals (CI), and *N* values (for each categorical climatic zone) indicate the number of rivers analysed. Compared to the zero residual value for which the ANN model perfectly fits the data, we obtained a significant *t*-test value for climatic zones IV (oceanic areas, overestimated mean value: *t*-test = -6.29, *P* < 0.001) and VI (continental areas, underestimated mean value: *t*-test = 2.11, *P* < 0.041) only. Thus, the additional contribution of climatic topography to global richness patterns appears to be negligible (*r* = 0.97, *P* < 0.0001) in comparison with the total effect of NPP, FR and SDA variables (*r* = 0.93, *P* < 0.0001).

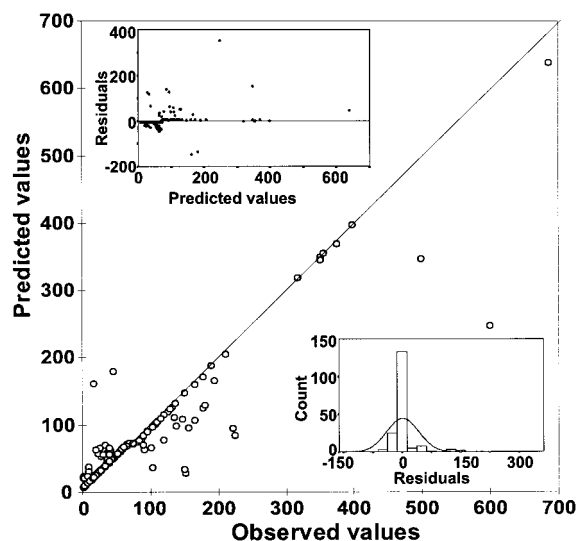


Figure 4 'Leave-one-out' cross-validation test for the 183 rivers analysed in this study. The relationship between predicted and observed SR values is shown; the correlation is highly significant (*n* = 183, *r* = 0.929, *P* < 0.0001) and most points perfectly fit the straight line for which predicted values equal observed ones (many points are superimposed on the figure). Top inset, the relationship shows some dependencies of residuals (*n* = 183, *r* = 0.259, *P* < 0.001) essentially due to some unfitted values in the model. Lower inset, frequency histogram of residuals with most values centred near zero (*n* = 183, mean = 1.40, s.d., ±40.82). Both insets illustrate conventional standard controls on statistics as for insets in Fig. 2.

topography (that is, the presence of a given river in one of the eight conventional climatic zones; see description of data) did not significantly contribute to the explanation of patterns of global-scale fish species richness (Fig. 3, inset), confirming that global fish species variability is not related to topography and latitudinal gradients, as was suggested by the historical hypothesis⁹. Our findings contradict a recent study in which North American mammal species richness was best predicted by a hierarchical sequence of limiting factors: that is, local energy availability was important in comparatively cold regions of high latitudes (Alaska and most of Canada), and topographic heterogeneity best explained species diversity for highly productive regions of the southern part of the continent (Southern Canada and United States)⁴. The main discrepancy between this previous work and our study on fish diversity lies in the different scales of the two analyses and in their geographical scope. Thus, we believe that large-scale fish-richness patterns are best explained by both energy availability and habitat diversity: the more energy available, the more fish species the aquatic environment can support; additionally, for regions with identical energy inputs, habitat heterogeneity may favour coexistence of more fish species.

The advantage of ANNs over conventional models stems from their ability directly to take into account nonlinear relationships, a common stumbling block when dealing with ecological systems^{19–21}, and so provide a more precise idea of the relationship between any influencing parameter and its dependent factor. Therefore, they are powerful models for forecasting purposes. A previous study using logarithmic transformation of variables succeeded in explaining up to 78% of the total variation in richness¹⁰, whereas the ANN method achieved a much higher level (~93%) with only three environmental parameters. Here, we show that large-scale species richness in riverine fish varies among regions as a nonlinear function of contemporary available energy in the system and local habitat heterogeneity in rivers. Characteristics of the ANN model parameters are practical for predicting and assessing global trends in biodiversity loss and habitat fragmentation. Important pervasive forms of environmental degradation due to human activities usually include source pollution, altered hydrological regimes (by dams, diversions and withdrawals) and habitat destruction^{22,23}. Consequences of such degradation are that many aquatic species are now threatened with extinction²⁴. To protect and maintain aquatic (and terrestrial) biodiversity, an understanding of the relationships between species and ecological processes that shape the entire ecosystem is essential^{25,26}. The development of artificial neural network models is a task of major importance in view of projections of global environmental change and the need for water-resource management. □

Methods

Description of data. Data employed in this study¹⁰ are based on a subsample of 183 plots (from a total of 292 rivers) for which all parameter values were available. We omitted the Amazon River basin, for which the richness value of ~2000 known fish species is subject to considerable error, and which, because of its extremely high value, may considerably bias the type of statistics used. We selected the most recent references and adjusted species number to account for extinction and introduction wherever possible. Only riverine fish species were included in the analyses and secondary or migratory euryhaline fishes were systematically withdrawn. Values for species richness (SR) refer to the total number of riverine fish species collected from the entire drainage basin, which corresponds to the current community richness per river. Three independent parameters were used as the best predictors of SR. There were: total surface of the drainage area (SDA) (generally taken from the literature, in km²); mean annual flow regime (FR) at the river mouth (also taken from the literature, in m³ s⁻¹, data were not available for all rivers, so we used only 183 rivers from the entire data set of 292); and net terrestrial primary productivity (NPP), which refers to the rate of energy flow through the plants of the region where a given river is located. We then tested the influence of contemporary climate

topography on SR values by analysing residual variations of richness obtained from the ANN model across the 8 conventional climatic zones (I to VIII). The rivers fall into the following zones: I, equatorial zone with very high annual precipitation; II, tropical summer-rainfall zone, with heavy rains in the summer and extreme drought during the cooler season; III, subtropical dry-zone of deserts, with very low rainfall; IV, Mediterranean transition zone with winter rainfall; V, warm-temperate climate zone with high humidity in summer; VI, temperate climate zone, with moderate humidity; VII, arid temperate climate zone of continental regions, with low rainfall; VIII, cold-temperate or boreal climate zone, with high precipitation.

Statistical analysis. ANN models are known for their capacity to process nonlinear relationships. We used one of the principles of ANNs, (the backpropagation algorithm²⁷), and a 'leave-one-out' cross-validation test (where each river sample is left out of the model formulation in turn and predicted once) to determine its performance (Fig. 4). This procedure is appropriate when the data set is quite small and/or when each sample is likely to have 'unique information' that is relevant to the regression model^{27,28}, as is frequently found in ecology. We used a typical three-layer feed-forward (3–5–1) ANN (Fig. 1). To determine the relative importance of the three input parameters, we used the procedure for partitioning the connection weights of the ANN model^{21,29,30}.

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Phantom sensations generated by thalamic microstimulation

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Many amputees have a sense of their missing 'phantom' limb¹⁻³. Amputation can alter the representation of the body's surface in the cerebral cortex⁴⁻¹⁴ and thalamus^{15,16}, but it is unclear how these changes relate to such phantom sensations. One possibility is that, in amputees who experience phantom sensations, the region of the thalamus that originally represented the missing limb remains functional and can give rise to phantom sensations even when some thalamic 'limb' neurons begin to respond to stimulation of other body regions. Here we use microelectrode recording and microstimulation during functional stereotactic mapping of the ventrocaudal thalamus in amputees to determine both the responses of the neurons to stimulation of the skin and the perceptual effects of electrical activation of these neurons. Thalamic mapping revealed an unusually large thalamic stump representation, consistent with the findings from animal experiments. We also found that thalamic stimulation in amputees with a phantom limb could evoke phantom sensations, including pain, even in regions containing neurons responsive to tactile stimulation of the stump. These findings support the hypothesis that the thalamic representation of the amputated limb remains functional in amputees with phantoms.

The severe phantom or stump pain that develops in some amputees can be treated with chronic electrical stimulation within the thalamus¹⁷. The stereotactic microelectrode mapping procedure associated with the implantation of thalamus-stimulating electrodes allows us to examine the properties of thalamic neurons and the sensory effects of thalamic microstimulation in awake patients. Therefore, to understand the thalamic mechanisms underlying phantom sensations, we examined thalamic neuronal responses and stimulation-induced sensations in six patients with postamputation pain. Four of these amputees experienced spontaneous phantom sensations but two had no phantom sensations.

In each amputee, microelectrode recordings and microstimulation were made in two or more trajectories through part of the thalamic ventrocaudal nucleus that represented the amputated limb, based on the presence of tactile receptive fields on the stump, and/or stimulation-evoked sensations (projected fields) in the stump/phantom region, and cells firing in a bursting pattern. Bursting is a common characteristic of neurons in damaged or deafferented regions¹⁸ of lateral thalamus. This mapping technique has previously shown that patients with no damage to the somatosensory system have a somatotopically organized representation of the body surface¹⁹ with a large representation of distal body parts, and with a close correspondence between the projected fields induced by microstimulation and the receptive fields of neurons at these sites^{20,21}.

In each 'phantom' patient, thalamic sites were encountered at which microstimulation evoked tingling, unpleasant and/or painful sensations in the phantom. Representative trajectories from three of these patients are shown (Figs 1, 2). The threshold currents for evoking phantom sensations were low (<25 μ A) and similar to those in 'normal' parts of the tactile ventrocaudal nucleus²¹. The projected fields frequently included only part of the phantom. At

sites of phantom projected fields, many cells had receptive fields on the stump, and other cells firing spontaneously, often in bursts, lacked a receptive field. In the three 'phantom' patients shown in Figs 1 and 2, the extent of proximal limb representation appeared to be abnormally large, as judged by the proportions of the total sample from each case (Table 1). The stump representation in the fourth 'phantom' patient appeared to be within the normal range found in non-amputees (Table 1). Phantom sensations could also be evoked by stimulation at some sites below the ventrocaudal nucleus in the medial lemniscus (Fig. 2). Thalamic mapping was also performed in and adjacent to the regions that normally represent the amputated limb in the two amputees who did not experience

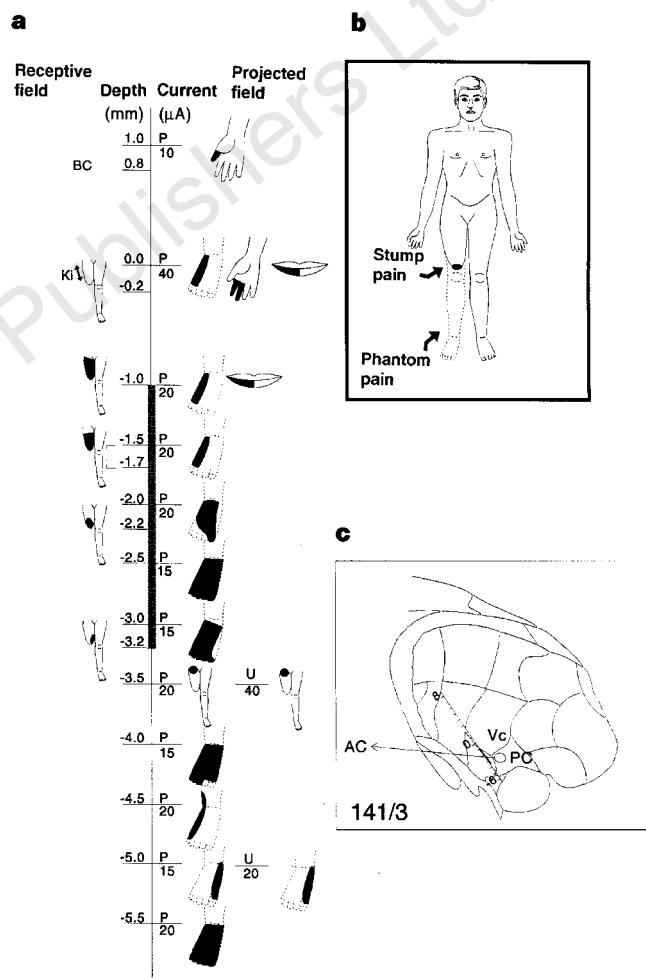


Figure 1 Results from patient 4 who had both stump and phantom pain after an amputation above the knee. **a**, A portion of the electrode trajectory reconstructed with neuronal receptive fields (left) and stimulation-evoked projected fields (right). Numbers indicate the threshold current (μ A) that produced the sensation, the quality of which is indicated by the letter: BC, bursting cell; Ki, kinaesthetic response; P, parasthesia; U, unpleasant. Receptive fields were based on responses to light tactile stimuli unless indicated. Note the sites of mismatches between receptive and projected fields such that thalamic stimulation evoked phantom sensations at sites where neurons responded to stump stimulation. **b**, The site of amputation and pain. **c**, The electrode trajectory on a sagittal map 19 mm lateral to the midline. The shaded bar in **a** and **c** indicates the region of tactile ventrocaudal nucleus (Vc) along the trajectory. The map is based on stereotactic coordinates and computer-scaled atlas maps based on the radiologically determined anterior and posterior commissures (AC and PC, respectively), and has not been corrected to fit the physiological findings. Recordings were not obtained below a depth of 3.2 mm. 141/2 refers to patient number and trajectory number.

Table 1 Individual patient histories and findings during thalamic mapping

Patient	Age* (years)	Sex	Amputation site	Time since amputation (years)†	Phantom‡	Location of sensations evoked by thalamic stimulation		Stump representation (% of sampled tactile ventrocaudal nucleus)§
						Stump	Phantom	
1	63	Male	above elbow	22	—	+	—	65
2	37	Female	below knee	13	—	+	—	17
3	49	Female	above knee	3	+	+	+	1
4	39	Male	below knee	6	+	+	+	13
5	64	Male	above elbow	13	+	+	+	15
6	62	Female	above knee	6	+	+	+	30

* Age at time of thalamic exploration.

† Time between amputation and thalamic exploration.

‡ Based on patient reports of spontaneous sensations in missing limb.

§ Representation (% of sampled length of tactile ventrocaudal nucleus) in 25 non-amputees (movement-disorder patients): 1 ± 1% upper arm; 4 ± 3% thigh.

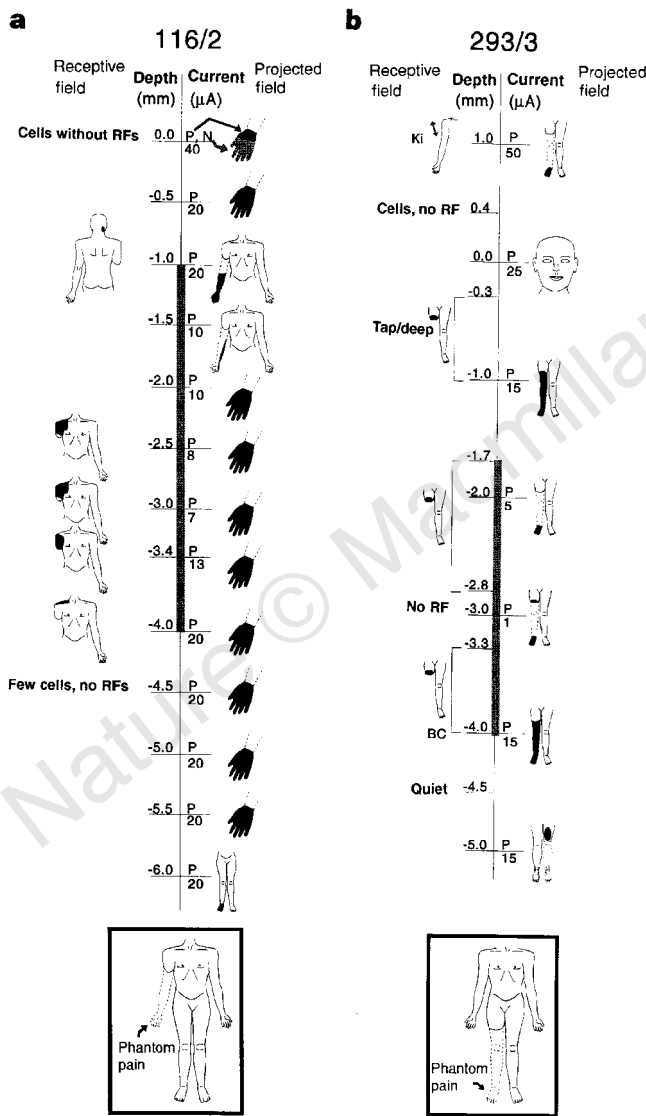


Figure 2 A portion of one electrode trajectory in each of two patients with phantom pain. The trajectories traversed a similar region of the ventrocaudal nucleus as shown in Fig. 1c. The sites of amputation and pain are shown below the trajectory for each patient. See Fig. 1 for explanation. Abbreviations: BC, bursting cell; Ki, kinaesthetic response; N, pain; P, parasthesia; PF, projected field; RF, receptive field. **a**, Results from patient 5, who had pain in a phantom arm and hand after an amputation above the elbow. Note the sites of stimulation-evoked phantom sensations, and neurons with either no receptive fields or responses to stump stimulation. Recordings were not obtained below a depth of 4.5 mm, where the electrode presumably was outside the tactile core of the ventrocaudal nucleus. **b**, Results from patient 6, who had phantom foot pain after an amputation above the knee. Note the thalamic stimulation-evoked sensations in the phantom foot and leg at sites of neuronal responses to stump stimulation. Recordings were not obtained below a depth of 4.5 mm, where the electrode was presumed to have left the tactile core of the ventrocaudal nucleus. 116/2 and 293/3 refer to patient and trajectory numbers.

the thalamic representation of the amputated limb remains functional, and implies that neuronal activity in this region gives rise to sensations perceived as originating from the missing part of the original limb. Furthermore, the large representation of the stump observed in most of these patients suggests that the representation of the stump region expanded into the original limb region of thalamus after the loss of distal limb inputs. These findings are consistent with those from studies of animals^{13,15,16,22} which demonstrated significant subcortical plasticity and expansion of somatotopically adjacent regions into deafferented regions. In many sites, recordings showed that neurons respond to tactile stimulation of the stump but thalamic stimulation evoked sensations on the phantom limb. On the basis of the large stump representation and its location where the distal limb should be represented, and the abnormal mismatch between receptive fields and projected fields, it is likely that these stump neurons used to respond to distal limb inputs. These findings suggest that, despite the appearance of new receptive fields, the activity of these thalamic neurons is still associated with the missing part of the limb they originally represented. This interpretation provides an explanation for clinical reports of phantom sensations, including dual percepts^{23,24}, evoked by tactile stimulation of an intact part of the body proximal to an amputation site^{10,23–28}.

Methods

Patient population. Data were obtained from three male and three female amputees, aged 37–64 years, who had undergone upper ($n = 2$) or lower ($n = 4$) limb amputation resulting in chronic pain in the stump (patients 1–3) or phantom (patients 4–6). One stump-pain patient had a non-painful phantom (patient 3). Thalamic exploration was performed 3–22 years after the amputation for implantation of chronic stimulating electrodes. Informed consent of procedures approved by the University of Toronto/Toronto Hospital Human Experimentation Committee was obtained from all patients.

any phantom sensations. Both patients had large representations of their stump (Table 1) but, in contrast to the ‘phantom’ patients, stimulation in this region evoked sensations only on the stump and other nearby areas of the body. A summary of the findings from each patient is provided in Table 1. These findings reveal that in the ‘phantom’ patients, even many years after loss of input from the limb, stimulation in the thalamus can still recreate sensations in the missing limb. This indicates that

Stereotactic recording and stimulation procedures. Standard methods were used for functional stereotactic procedures²⁹. Microelectrode recordings and stimulation were performed with either glass- or parylene-coated tungsten microelectrodes. Our initial target was chosen near the ventral border (at or close to the line between the anterior and posterior commissures of the ventrocaudal nucleus, in a sagittal plane 14–15 mm lateral to the midline. Subsequent electrode trajectories were appropriate for the patient's site of pain. Each electrode trajectory was made through the thalamus in a parasagittal plane. The standard search procedure during each penetration consisted of recording the neuronal activity continuously as the electrode was advanced. At sites of spontaneous neuronal activity, stimuli were applied to the joints (passive and/or active movements), muscles (squeeze) and skin (brush and hot and cold probes) to delineate receptive fields. At intervals of 0.5–1 mm, the microelectrode was used to deliver brief trains of electrical stimuli (1 s, 300-Hz trains of 0.2 ms monophasic (tip negative) pulses of up to 100 μ A) to determine the threshold for sensation, and the patient was asked to describe the location (projected field) and quality of any evoked sensation. To assess abnormalities in body representations in each amputee, the length along each trajectory that represented the stump region was summed and expressed as a percentage of the total sampled length of tactile ventrocaudal nucleus in that patient. The data obtained from non-amputee patients with movement disorders was sampled from a comparable region of the ventrocaudal nucleus²².

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Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein

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Point mutations in the presenilin-1 gene (*PS1*) are a major cause of familial Alzheimer's disease. They result in a selective increase in the production of the amyloidogenic peptide amyloid- β (1–42) by proteolytic processing of the amyloid precursor protein (APP)^{1–4}. Here we investigate whether PS1 is also involved in normal APP processing in neuronal cultures derived from PS1-deficient mouse embryos. Cleavage by α - and β -secretase⁵ of the extracellular domain of APP was not affected by the absence of PS1, whereas cleavage by γ -secretase of the transmembrane domain of APP was prevented, causing carboxyl-terminal fragments of APP to accumulate and a fivefold drop in the production of amyloid peptide. Pulse-chase experiments indicated that PS1 deficiency specifically decreased the turnover of the membrane-associated fragments of APP. As in the regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor⁶, PS1 appears to facilitate a proteolytic activity that cleaves the integral membrane domain of APP. Our results indicate that mutations in *PS1* that manifest clinically cause a gain of function and that inhibition of PS1 activity is a potential target for anti-amyloidogenic therapy in Alzheimer's disease.

Mice deficient in the expression of PS1 (*PS1*^{−/−} mice) were generated by homologous recombination (see Methods). We confirmed that homozygous *PS1*^{−/−} mice die late in embryogenesis^{7,8}. Growth of the embryo was severely retarded, being most evident in the caudal region as a stubby tail. It has been proposed that this phenotype is a result of disturbed Notch signalling^{7,8}. The embryonic lethality of *PS1*^{−/−} mice precludes further analysis of the possible effects on APP metabolism in living animals. Therefore, brain cultures were generated from living *PS1*^{−/−} embryos at day 14 post coitum (pc) according to protocols used previously for hippocampal neurons^{9–12}. Cell yields were $7.6 (\pm 1.6) \times 10^6$ cells for *PS1*^{+/+} ($n = 6$), $7.8 (\pm 1.3) \times 10^6$ cells for *PS1*^{+/−} ($n = 7$), and $6.0 (\pm 1.3) \times 10^6$ cells for *PS1*^{−/−} embryos ($n = 3$). Cultures derived from littermate embryos with the different genotypes were morphologically indistinguishable. They contained neuronal cells almost exclusively, as evaluated by phase-contrast and immunofluorescence microscopy using antibodies against Tau, Map2 and GFAP proteins (results not shown). *PS1*^{+/+} and *PS1*^{−/−} cultures were metabolically labelled and amyloid peptide and carboxy-terminal fragments from endogenously expressed mouse APP were analysed by immune precipitation (Fig. 1b, c). The strong inhibition of secretion of amyloid- β peptide and of the p3 fragment (generated by combined β - and γ -secretase and α - and γ -secretase proteolytic

activity⁵, respectively; Fig. 1a), and the accumulation of C-terminal fragments of APP in *PS1*^{-/-} neurons, demonstrates a direct role for PS1 in the amyloidogenic processing of mouse APP. Quantitative analysis of this effect was difficult because of the low signals obtained with endogenous mouse APP. We therefore expressed human wild-type APP and human APP containing the London (Val at position 642 to Ile) or Swedish (Lys at position 595 to Asn and Met 596 to Leu) clinical mutations in the neurons by using recombinant Semliki Forest virus (SFV)⁹⁻¹². Using a panel of well characterized antibodies against APP the proteolytic processing of APP was analysed in *PS1*^{-/-} cells (Fig. 1a). The catabolic intermediates visualized here have been previously identified by labelled-amino-acid sequencing¹¹. Similar amounts of APP holoprotein (100K–140K) expression were obtained in *PS1*^{+/+}, *PS1*^{+/-} and *PS1*^{-/-} neuronal cultures (Fig. 1d). Secretion of APP ectodomain was the same in *PS1*^{-/-} and in *PS1*^{+/+} littermate controls (Fig. 1e), indicating that α - and β -secretase processing of APP was normal. This was confirmed using antibodies against amino-acid residues 595–612 (R1736; ref. 13) and residues 595–596 (ref. 14) of APP, which are specific for α - and β -secretase-cleaved APP ectodomain, respectively (Fig. 1f). Human amyloid- β peptide secretion

detected by anti-APP597–612 (ref. 9) was significantly lower from the *PS1*^{-/-} than from *PS1*^{+/+} cells (Fig. 1g and Table 1). In control experiments using antibody 1282 (ref. 14), we confirmed the strong decrease in p3 secretion from virally transfected cells (results not shown), which was similar to that from uninfected cells (Fig. 1b). The fivefold decrease in amyloid peptide secretion was not accompanied by a concomitant accumulation of cell-associated amyloid peptide (Fig. 1h). In contrast, a twofold increase in β -secretase-cleaved and a fivefold increase in α -secretase-cleaved C-terminal fragments was observed in the cell extracts (Fig. 1d, h; Table 1).

In cultures derived from *PS1*-heterozygous embryos, either no or only weak effects on APP processing were observed (Fig. 1), in accordance with their normal phenotype^{7,8}. APP containing either the London or the Swedish mutations that cause early-onset familial Alzheimer's disease (Fig. 1) underwent the same abnormal processing: a two- to fivefold accumulation of α - and β -secretase-cleaved C-terminal APP stubs in the cell extracts, a two- to fivefold inhibition of amyloid peptide secretion into the culture medium, and either no or only a marginal effect on secretion of APP ectodomain into the medium (Table 1).

The apparent paradox of an increase in C-terminal fragments in

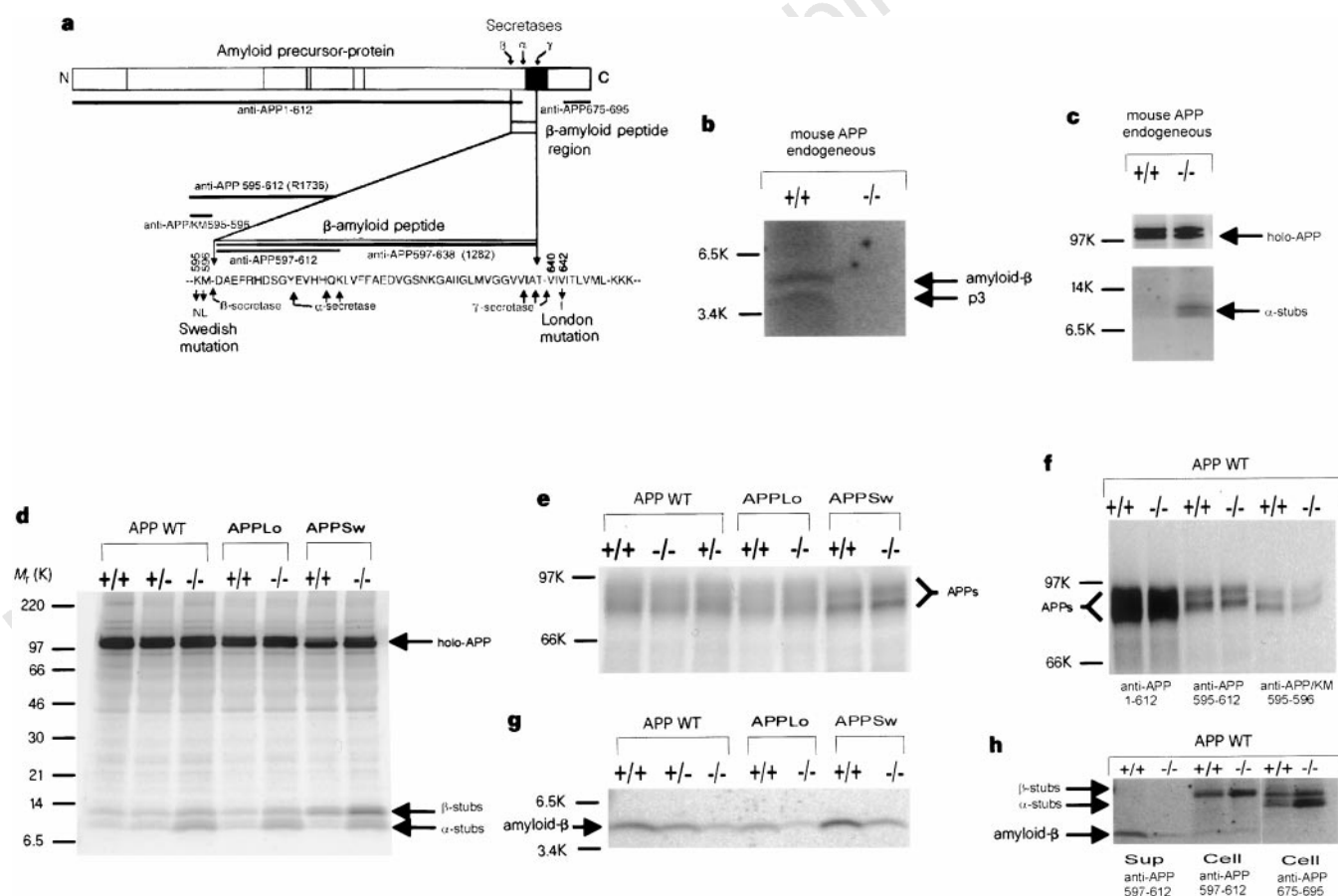


Figure 1 Processing of amyloid precursor protein. **a**, Structure of APP and the regions targeted by antibodies used in this study^{9-14,29}. **b**, Amyloid- β and p3 peptide were immunoprecipitated from the medium of metabolically labelled neurons (for 18 h) with APP597–638 (R1282; ref. 13). **c**, Cell-associated APP (holo-APP) and C-terminal fragments were precipitated with APP675–695 (ref. 9) from cell extracts (normalized for TCA-precipitable label). Mainly α -secretase stubs are generated from mouse APP^{9,11}. (See **a** for the cleavage sites¹¹). **d**, Cell-associated APP fragments precipitated with anti-APP675–695 from neurons expressing human wild-type APP, or APP containing either the London (APP Lo) or the Swedish (APP Sw) mutation after infection with recombinant SFV⁹⁻¹². For characterization of the β - and α -secretase-cleaved fragments, see ref. 11. **e**, Secreted APP ectodomain (APPs) was immunoprecipitated with anti-APP1–612 (ref. 29); the

doublet appearance represents the different sialic acid content¹¹. Note the small increase in mobility of APP Sw (APPs β generated by β -secretase) compared to APPs α . **f**, Soluble APPs, APPs α and APPs β precipitated with anti-APP1–612, anti-APP595–612 (R1736), and anti-APP595–596 (ref. 14), respectively. **g**, Secreted amyloid- β peptide precipitated with anti-APP597–612 (which does not react with p3)⁹. **h**, Amyloid- β peptide precipitated from medium ('Sup') and cell extracts ('Cell') using anti-APP597–612. β -Secretase-generated fragments (lanes 3, 4) coprecipitate with small amounts of cell-associated amyloid- β peptide. The intensity of the signals obtained for intracellular amyloid peptide is too low for reliable analysis (but see Fig. 3). Fragments generated by α - and β -secretase but no amyloid- β peptide are precipitated with anti-APP675–695, demonstrating the specificity of the signals in the previous two lanes.

cell extracts without a concomitant increase in APP ectodomain in the medium can be explained if PS1 is involved in γ -secretase processing of APP after cleavage by α - and β -secretase, resulting in turnover of these fragments. We verified this by pulse-chase experiments, which showed that newly generated C-terminal fragments of APP accumulated during four hours of chase in $PS1^{-/-}$ cells. In $PS1^{+/+}$ neurons, these fragments must be rapidly turned over as they were barely detectable at any time point (Fig. 2). The production of both amyloid- $\beta(1-40)$ and amyloid- $\beta(1-42)$ peptides in the medium of $PS1^{-/-}$ and wild-type neuronal cultures was measured by enzyme-linked immunosorbent assay (ELISA) and shown to be decreased 3.6- and 3.2-fold, respectively (Fig. 3). The two putative γ -secretases¹⁵⁻¹⁷ appear to be equally affected by the $PS1$ null mutation. We intend to investigate whether PS2 is responsible for the residual secretion of amyloid peptide in $PS1^{-/-}$ cultures by using $PS2^{-/-}$ mice and double-knockout embryos once they have been generated.

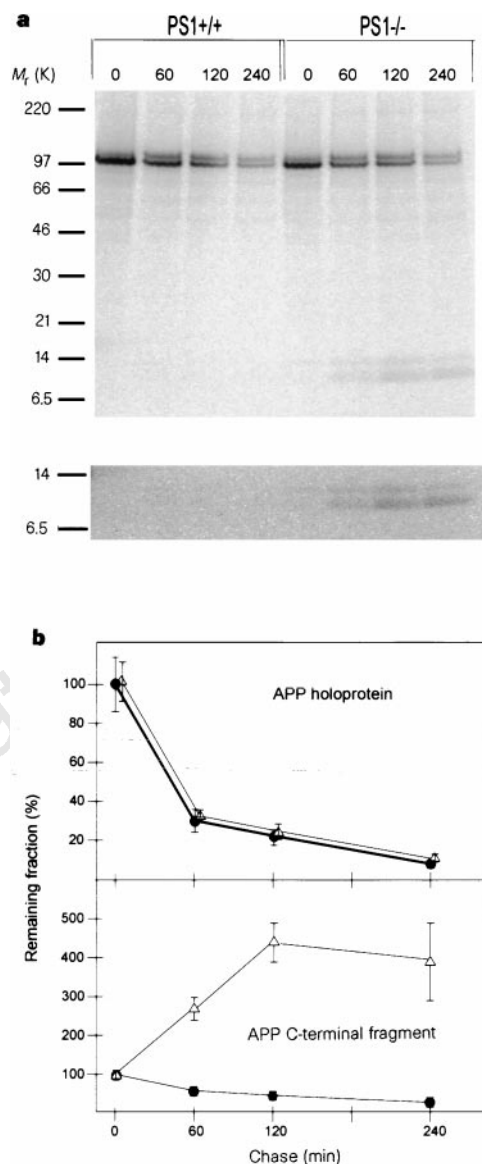


Figure 2 **a**, Pulse-chase experiment demonstrating similar turnover rates for APP holoprotein in $PS1^{+/+}$ and $PS1^{-/-}$ cultures. The lower half of the gel (after amplification of the signals) demonstrates the turnover of the C-terminal fragments. Pulse labelling, 30 min; chase, 60, 120 and 240 min. **b**, Quantitative analysis. All data are normalized¹² to the signal obtained at the end of the pulse labelling for APP holoprotein (top) and the carboxy-terminal fragments (bottom) from $PS1^{+/+}$ (●) and $PS1^{-/-}$ (△) cultures. Bars indicate s.e.m. ($n = 3$).

Table 1 Different APP fragments in $PS1^{-/-}$ and $PS1^{+/+}$ cultures

	APP WT	APP Lo	APP Sw
Cleaved stubs (β -secretase)	2.1 ± 0.4	2.3 ± 0.1	2.1 ± 0.3
Cleaved stubs (α -secretase)	5.4 ± 0.9	5.9 ± 1.6	4.6 ± 0.2
APP ectodomain (total)	1.0 ± 0.2	1.5 ± 0.1	0.9 ± 0.1
Amyloid- β peptide (total)	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0

All APP fragments (Fig. 1) were quantified by phosphorimaging in arbitrary densitometric volume units and normalized to the volume of APP holoprotein in the same culture to compensate for differences in cell numbers and infection¹². The values obtained from $PS1^{-/-}$ cultures were divided by the values obtained in the $PS1^{+/+}$ cultures and this ratio is shown as the mean $\pm$ s.e.m. of three independent experiments. APP WT, wild-type APP; APP Lo, APP containing the London mutation (Val642 $\rightarrow$ Ile); APP Sw, APP containing the Swedish mutations (Lys595 $\rightarrow$ Asn and Met596 $\rightarrow$ Leu).

Our results show that PS1 is involved in γ -secretase-mediated proteolytic cleavage of the C-terminal transmembrane fragments of APP after their generation by α - and β -secretase(s). PS1 is the only protein identified so far to be involved in one of the steps in APP processing by secretases. This mechanism may extend to other membrane-spanning proteins and suggests a physiological function for presenilin-1. Although PS1 itself may be (one of) the elusive γ -secretase(s), this is unlikely given the absence of sequence identity and lack of structural homology between PS1 and any proteinase domain known¹. The residual amyloid-peptide secretion found for $PS1^{-/-}$ neurons suggests that PS1 is not the enzyme itself, but a regulatory cofactor in this processing step. The demonstration that APP and presenilins interact with one another fits this hypothesis^{18,19}. The structural and functional similarity of PS1 and SCAP⁶ may be significant: they both span the endoplasmic reticulum membrane 6 to 8 times²⁰⁻²³ and both are involved in the cleavage of integral membrane proteins (APP and SREBP, respectively). PS1 and SCAP may represent a new class of chaperones that mediate or control access of proteinases to the transmembrane domain in the case of APP and to the luminal-loop domain in the case of SREBP. An alternative explanation that PS1 is involved in the transport of the membrane-anchored C-terminal APP fragments towards the γ -secretase(s)-containing subcellular compartment does not necessarily contradict this hypothesis. Our data strongly support the idea that clinical mutations in $PS1$ result in a gain of the function of PS1, and are not a haplotype insufficiency as was inferred from complementation experiments in *Caenorhabditis elegans* deficient in *sel12*, the worm's homologue of PS1 (refs 24,

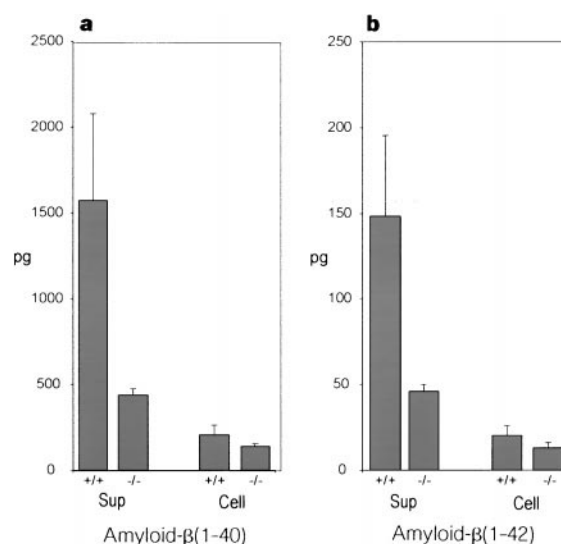


Figure 3 Assessment of the **a**, amyloid- $\beta(1-40)$ -, and **b**, amyloid- $\beta(1-42)$ -peptide contents in supernatants ('Sup') and cell extracts ('Cell') of $PS1^{+/+}$ and $PS1^{-/-}$ littermate neuronal cultures. The total amounts (in picograms) of peptide per culture dish are shown as mean $\pm$ s.d. ($n = 4$).

25). Our results indicate that production of amyloid peptide in neurons might be decreased by inhibiting PS1 function. With the proviso that PS1 function might be essential in adult brain, to be evaluated in conditionally targeted PS1 mice, PS1 may be a suitable target for anti-amyloidogenic therapy in Alzheimer's disease. □

Methods

Additional information describing the generation and characterization of the PS1^{-/-} mice is available on our website <http://www.med.kuleuven.ac.be/legtegg/>.

The preparation of recombinant Semliki Forest virus stocks and the infection of neuronal cultures have been described⁹⁻¹². Virus was diluted in culture medium and added to 3–5-day-old neuronal cultures. After viral adsorption (for 1 hour), the virus solution was replaced by normal medium. After 2 h, culture was continued in methionine-free medium containing 200 $\mu\text{Ci ml}^{-1}$ of [³⁵S]methionine. After 4 h of metabolic labelling, cell extracts and supernatants were processed as described⁹⁻¹². Radioactive bands were measured as densitometric volumes using phosphorimaging. Immunoprecipitated material was resolved in 10–20% Tris–tricine gels or in 6% Tris–glycine gels for the analysis of APP fragments.

The sandwich-type ELISA^{26,27} is based on capture antibodies 21F12 (specific for amyloid- β (1–42)) and FCA3340 (specific for amyloid- β (1–40))²⁸ and biotinylated reporter antibody 3D6. Synthetic peptides (Bachem) were used as standards. Controls showed no crossreactivity of the assay with shorter or longer forms of the peptides.

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NIP domain prevents N-type inactivation in voltage-gated potassium channels

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Shaker-related voltage-gated K⁺ (K_v) channels^{1,2} are assembled from ion-conducting K_v α subunits, which are integral membrane proteins, and auxiliary K_v β subunits. This leads to the formation of highly diverse heteromultimeric K_v channels that mediate outward currents with a wide range of time courses for inactivation. Two principal inactivation mechanisms have been recognized¹: C-type inactivation correlated with carboxy-terminal K_v α -subunit structures³, and N-type inactivation conferred by 'ball' domains in the amino termini of certain K_v α ^{4,5} and K_v β ⁶ subunits. Assembly of heteromultimers with one or more K_v α ^{4,7}-and/or K_v β ⁶ ball domains appears to be an essential principle of the generation of A-type K_v channel diversity. Here we show that, unexpectedly, the presence of K_v α - or K_v β -ball domains does not dominate the gating phenotype in heteromultimers containing K_v1.6 α subunits. These heteromultimers mediate non-inactivating currents because of the dominant-negative activity of a new type of N-type inactivation-prevention (NIP) domain present in the K_v1.6 amino terminus. Mutations in the NIP domain lead to loss of function, and its transfer to another K_v α subunit leads to gain of function. Our discovery of the NIP domain, which neutralizes the activity of K_v α - and K_v β -inactivation gates, establishes a new determinant for the gating behaviour of heteromultimeric K_v channels.

Unlike in other K_v1 α /K_v1.1 heteromultimers^{6,8}, such as K_v1.5/K_v1.1 ($\tau_h = 1.5 \pm 0.2$ ms, $n = 6$; Fig. 1a), K_v1.1 did not confer rapid N-type inactivation to non-inactivating K_v1.6 channels ($n = 5$; Fig. 1b). Coexpression of K_v1.1 with K_v1.6, however, shifted the voltage-dependent activation of the K_v1.6 outward currents ($V_{1/2} = 15 \pm 2.7$ mV, slope factor 12 ± 1.1 mV, $n = 4$) to more negative potentials ($V_{1/2} = 4.9 \pm 4.9$ mV, slope factor 8.9 ± 1.2 mV, $n = 5$). Similar shifts in the voltage dependence of activation had been observed with K_v2 subunits, which do not possess a ball domain, when coexpressed with K_v1 α subunits^{8,9}. Thus K_v1.1 apparently influenced K_v1.6 channel gating like a K_v β subunit without an inactivation gate. This implies that K_v1.6/K_v1.1 heteromultimers were formed, as suggested by co-immunoprecipitation studies¹⁰. The K_v1.6 N terminus contains a conserved functional K_v1-binding site^{11,12}, as indicated by protein overlay binding

assays. *In vitro* translated [35 S]K β 1.1 protein bound to immobilized K α 1.6 N terminus expressed as a fusion protein in *Escherichia coli* (Fig. 1c). In comparison to the K α 1.5 N terminus, the signal intensity was relatively light, but was similar to that for [35 S]K β 1.1 binding to the K α 1.1 N terminus¹².

According to the 'ball and chain' model of N-type inactivation^{4,13}, the N-terminal K α 1.1 ball domain occludes K α channels by binding to a temporary docking site (the ball receptor) at the cytoplasmic side of the pore^{13,14}. The presence of a functional K α 1.6 ball receptor was demonstrated in inside-out patches containing K α 1.6 channels (Fig. 1d). Bath application of K β 1.1 (ref. 6) or *Shaker* K α (ref. 13) ball peptide induced rapid inactivation. The time constants for K α 1.6 inactivation (K β 1.1 peptide, $\tau_h = 3.7 \pm 1.5$ ms, $n = 3$;

Shaker peptide, $\tau_h = 12.9 \pm 1.9$ ms, $n = 3$) were very similar to those obtained for K α 1.5 (K β 1.1 peptide, $\tau_h = 3.2 \pm 1.2$ ms, $n = 3$; *Shaker* peptide, $\tau_h = 9.7 \pm 3.3$ ms, $n = 3$). We therefore hypothesized that N-type inactivation in K α 1.6/K β 1.1 heteromultimers was obstructed by an N-type inactivation-prevention (NIP) domain, rather than by a dysfunctional K α 1.1 binding site or ball receptor.

The NIP domain resides in the K α 1.6 N terminus, as chimaeric K α 1.5/K α 1.6 channels with a K α 1.5 N terminus (K α 1.5NM1.6C; K α 1.5N1.6MC, see Fig. 2a, b) mediated fast inactivating currents (Fig. 2a, b) in the presence of K β 1.1 (K α 1.5NM1.6C, $\tau_h = 5.5 \pm 0.8$ ms, $n = 4$; K α 1.5N1.6MC, $\tau_h = 5.0 \pm 1.0$ ms, $n = 3$). In further chimaeric constructs the C-terminal part of the K α 1.6 N terminus, which contains the α -subunits tetramerization (T1)¹⁵ and

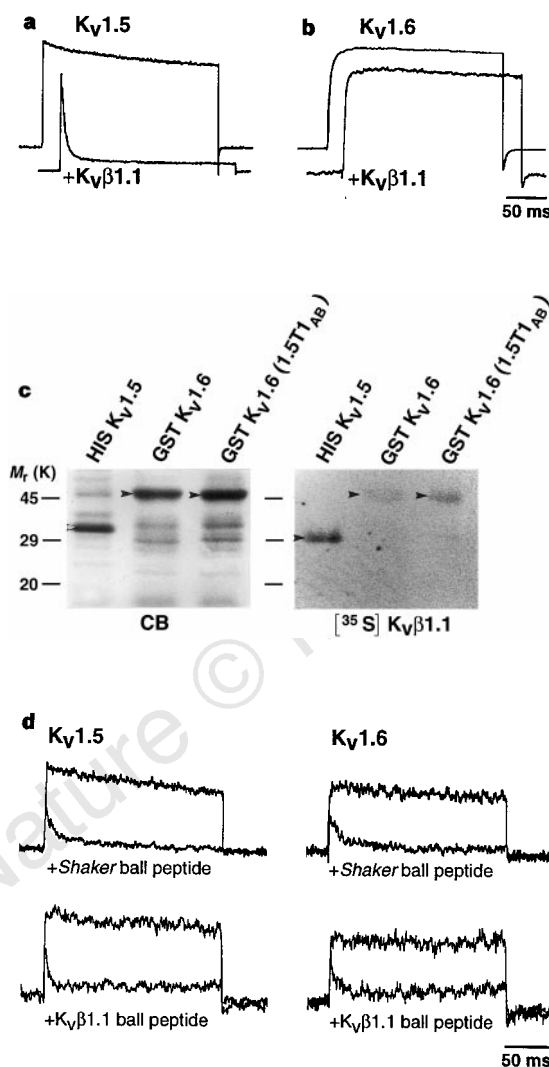


Figure 1 Inactivation properties of K α 1.6 channels are not altered by the presence of K β 1.1 subunit. **a, b**, Scaled outward currents elicited by depolarizing pulses to +60 mV from a holding potential of -80 mV in whole-cell patch-clamp recordings. Chinese hamster ovary (CHO) cells were injected with cDNA expression vectors coding for K α 1.5, K α 1.6 and K β 1.1, as indicated. The K β 1.1 subunit accelerated the inactivation of currents mediated by K α 1.5 but not K α 1.6. **c**, K β 1.1 binding to K α 1.5, K α 1.6 and K α 1.6(1.5T1_{AB}) N termini expressed as glutathione S-transferase (GST) or His-tagged (HIS) fusion proteins in *E. coli*. Coomassie blue-stained SDS-polyacrylamide gel (CB) of total *E. coli* lysates and autoradiograms of the corresponding nitrocellulose membrane blots incubated with *in vitro* translated, 35 S-labelled K β 1.1 probe ([35 S]K β 1.1). Fusion proteins are marked with arrows. Relative molecular mass (M_r) markers are shown. **d**, Normalized K α 1.5 and K α 1.6 currents recorded from inside-out patches before and during application of 100 μ M *Shaker* K α 1.3 or K β 1.1 ball peptide⁶ as indicated.

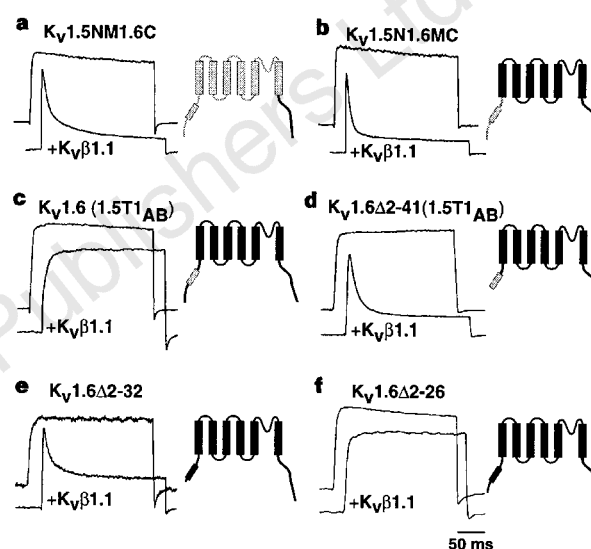


Figure 2 Localization of the N-type inactivation prevention (NIP) domain to the K α 1.6 N terminus. Scaled outward currents elicited by depolarizing pulses to +60 mV from a holding potential of -80 mV in whole-cell patch-clamp recordings. CHO cells were injected with cDNA expression vectors coding for: **a**, K α 1.5NM1.6C; **b**, K α 1.5N1.6MC; **c**, K α 1.6(1.5T1_{AB}); **d**, K α 1.6Δ2-41(1.5T1_{AB}); **e**, K α 1.6Δ2-32; **f**, K α 1.6Δ2-26. Currents were recorded in the absence or presence of K β 1.1. Chimaeric K α 1.5/K α 1.6 and truncated K α 1.6 subunits are shown schematically. K α 1.5 sequences are shown grey, K α 1.6 sequences in black. Rectangular bars, hydrophobic membrane-spanning segments; small bar, cytoplasmic α -subunit tetramerization (T1)¹⁵ and K β 1.1-binding domains¹¹² (amino acids 42-149 for K α 1.6; 112-216 for K α 1.5).

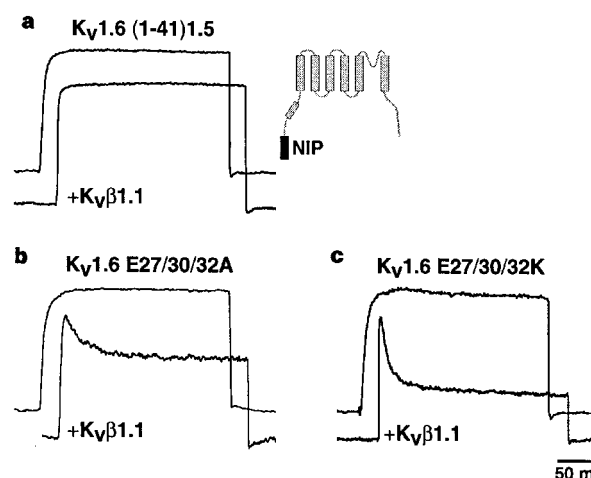


Figure 3 Gain- and loss-of-function NIP domain mutants. CHO cells were injected with cDNA expression vectors coding for: **a**, K α 1.6(1-41)1.5; **b**, K α 1.6 E27/30/32A; **c**, K α 1.6 E27/30/32K. Scaled outward currents recorded $\pm$ K β 1.1, as in Fig. 1. See Fig. 2 for explanation of chimaeric NIP, K α 1.5 subunit diagram.

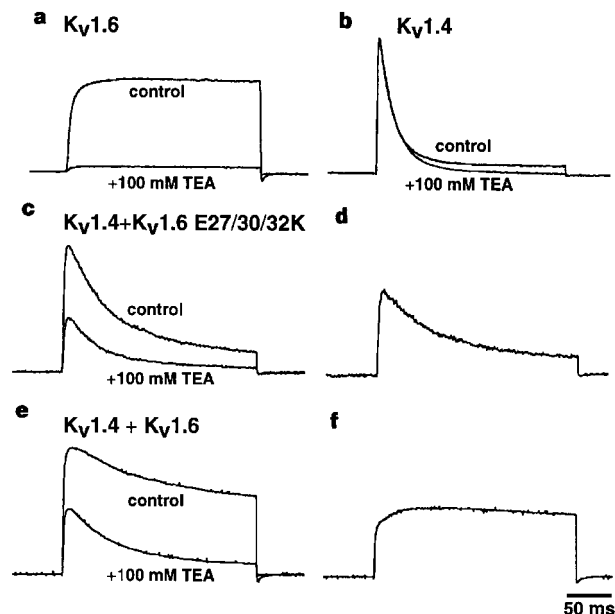


Figure 4 The NIP domain overrides N-type inactivation in heteromultimeric $K_v1.4/K_v1.6$ channels. CHO cells were injected with cDNA expression vectors coding for: **a**, $K_v1.6$; **b**, $K_v1.4$; **c**, $K_v1.4$ and $K_v1.6$ E27/30/32K; **e**, $K_v1.4$ and $K_v1.6$. Scaled outward currents recorded (as in Fig. 1) before (control) and after bath application of 100 mM TEA. **c**, After bath application of TEA, $K_v1.4/K_v1.6$ E27/30/32K current is reduced to $58 \pm 17\%$, $n = 4$. **d**, TEA-sensitive $K_v1.4/K_v1.6$ E27/30/32K current calculated as the difference between control and current remaining in 100 mM TEA. **e**, $K_v1.4/K_v1.6$ current is reduced to $62 \pm 19\%$ in 100 mM TEA, $n = 3$. **f**, TEA-sensitive $K_v1.4/K_v1.6$ current was calculated as the difference between control and current remaining in 100 mM TEA bath.

$K_v\beta$ -binding^{11,12} domains, was replaced by that of $K_v1.5$. Overlay binding experiments showed that a fusion protein containing a $K_v1.6(1.5T1_{AB})$ N terminus bound [³⁵S] $K_v\beta1.1$ (Fig. 1c), but $K_v\beta1.1$ did not confer N-type inactivation to $K_v1.6(1.5T1_{AB})$ channels (Fig. 2c). However, when the first 41 amino-acid residues of the $K_v1.6$ N terminus were deleted ($K_v1.6\Delta2-41(1.5T1_{AB})$), A-type K_v channels were obtained ($\tau_h = 8.3 \pm 1.3$ ms, $n = 4$; Fig. 2d). Also, coexpression of $K_v\beta1.1$ and $K_v1.6$ with truncated N terminus ($K_v1.6\Delta2-32$) produced rapidly inactivating currents ($\tau_h = 11.1 \pm 2.4$ ms, $n = 4$; Fig. 2e). In contrast, when we deleted only the first 26 N-terminal amino acids ($K_v1.6\Delta2-26$), coexpression with $K_v\beta1.1$ yielded non-inactivating currents ($n = 4$; Fig. 2f). Thus the NIP domain was close to the $K_v1.6$ N-terminal end, the sequence of which is not conserved among $K_v1\alpha$ subunits¹⁶.

Transplantation of the $K_v1.6$ N-terminal end to $K_v1.5$ ($K_v1.6(1-41)1.5$) generated K_v channels that, unlike $K_v1.5$ wild-type channels, were not inactivated by $K_v\beta1.1$ ($n = 5$; Fig. 3a). Thus the NIP domain may function as an independent inhibitory module for N-type inactivation. Within the NIP domain a small negatively charged sequence (EFQEAE) between amino acids 27 and 32 appeared to be important for function. Replacement of glutamic acids Glu 27, Glu 30 and Glu 32 by alanine ($K_v1.6$ E27/30/32A) or lysine ($K_v1.6$ E27/30/32K) yielded mutant $K_v1.6$ channels that mediated outward currents with a rapidly inactivating component ($K_v1.6$ E27/30/32K, $\tau_h = 20.1 \pm 1.1$ ms, $n = 5$, ~40% of peak amplitude; $K_v1.6$ E27/30/32A, $\tau_h = 10.9 \pm 3.4$ ms, $n = 5$, ~60% of peak amplitude; Fig. 3b, c). In contrast, replacement of Phe 28 and Glu 29 by alanine ($K_v1.6$ F28Q29/A) did not attenuate NIP activity (data not shown).

We tested with $K_v1.4/K_v1.6$ heteromultimers whether the NIP domain also inhibits $K_v1\alpha$ subunit-mediated N-type inactivation. Because homomultimeric $K_v1.6$ channels mediated non-inactivating tetramethylammonium (TEA)-sensitive¹⁷ (Fig. 4a)

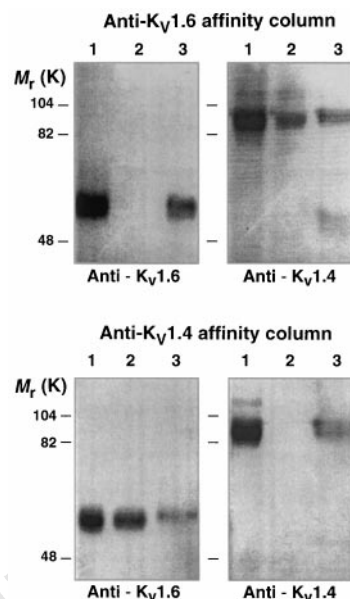


Figure 5 Heteromultimeric rat brain K_v channels contain $K_v1.4$ and $K_v1.6$ subunits. Solubilized rat membrane protein was loaded onto either an anti- $K_v1.6$ (top) or an anti- $K_v1.4$ (bottom) affinity column. Equal amounts of starting material (lane 1), flow through (lane 2) and eluate (lane 3) were analysed on western blots and immunostained with anti- $K_v1.6$ and anti- $K_v1.4$ antibodies as indicated. M_r standards are indicated. Note that the immunostained bands correspond to the previously published apparent molecular weights of the $K_v1.6$ (M_r 58K) and $K_v1.4$ subunits (M_r 94K)²⁰.

and homomultimeric $K_v1.4$ channels, rapidly inactivating ($\tau_h = 27.4 \pm 6.4$ ms, $n = 4$) TEA-resistant¹⁸ outward currents (Fig. 4b), coexpression of $K_v1.4$ and $K_v1.6$ subunits should generate heteromultimeric TEA-sensitive K_v channels¹⁹ that inactivate rapidly in the absence (Fig. 4d), but not in the presence, of a functional NIP domain (Fig. 4f). Indeed, coexpression of $K_v1.4$ and $K_v1.6$ E27/30/32K (Fig. 4c) gave rise to transient TEA-sensitive A-type currents ($\tau_h = 32.0 \pm 4.9$ ms, $n = 4$; Fig. 4c, d). In contrast, coexpression of $K_v1.4$ and wild-type $K_v1.6$ resulted in the expression of TEA-sensitive, non-inactivating outward currents ($n = 3$; Fig. 4e, f); they activated at more negative potentials ($V_{1/2} = -2.9 \pm 1.4$ mV, $n = 3$) than $K_v1.6$ currents (Fig. 1a). Accordingly, the activation kinetics of $K_v1.4/K_v1.6$ currents ($\tau = 3.8 \pm 1$ ms, $n = 3$) at 0 mV were faster than those of $K_v1.6$ ($\tau = 25 \pm 6$ ms, $n = 3$). The data demonstrate that the NIP domain also prevents N-type inactivation in $K_v1.4/K_v1.6$ heteromultimers. *In situ* hybridization and immunohistochemical localization studies suggested that $K_v1.4$ and $K_v1.6$ proteins may coexist in several types of neurons in rat brain, for example in the hippocampal CA3 region²⁰. Direct association of $K_v1.4$ and $K_v1.6$ subunits in native heteromultimeric K_v channels was demonstrated in immunoaffinity experiments using anti- $K_v1.4$ and anti- $K_v1.6$ antibodies. Solubilized rat-brain membrane proteins were bound to either an anti- $K_v1.4$ antibody or an anti- $K_v1.6$ antibody affinity column in non-denaturing conditions (Fig. 5). Anti- $K_v1.4$ antibodies consistently retained not only $K_v1.4$ protein, as detected by the immunoblots of the column eluate, but also smaller amounts of $K_v1.6$. Conversely, eluates of the anti- $K_v1.6$ immunoaffinity column contained $K_v1.4$ protein as well as $K_v1.6$ protein.

The results show that $K_v1.4$ - or $K_v\beta1.1$ -ball domains did not rapidly inactivate heteromultimeric K_v channels containing $K_v1.6$ subunits (NIP⁺ K_v channels), although these channels possess a functional ball receptor^{13,14} and a conserved $K_v\beta1$ -binding domain^{11,12}. Deletion or mutation of the NIP domain restored

N-type inactivation. It is likely that the NIP domain neutralizes ball activity in a dominant-negative manner, suggesting a functional interaction for NIP and ball domains in NIP⁺ K_v channels. Future work may be directed to understanding the molecular and structural details of the interplay of the two antagonistic N-terminal domains in determining the gating phenotype of K_v channels. □

Methods

Construction of expression clones. A cDNA fragment coding for K_v1.6 N terminus (nucleotides 431–955, amino acids 1–175, accession no. X17621) was ligated with BamHI/EcoRI-digested pGEX-2T. Construction of plasmid for expression of K_v1.5 N terminus, induction of protein expression in *E. coli*, and protein overlay binding assay were as described¹². For electrophysiological experiments, cDNAs^{6,17,18} coding for K_v1.4, K_v1.5, K_v1.6 and K_vβ1.1 were cloned into pcDNA3 expression vector (Invitrogen).

In vitro mutagenesis. Chimaeras between K_v1.5 and K_v1.6 were obtained by overlap polymerase chain reaction (PCR)²¹. Wild-type fragments were replaced by chimaeric fragments using appropriate restriction enzymes. N-terminal deletion mutants were constructed by PCR using sense primers that introduce a HindIII restriction site, a Kozak consensus sequence²², and a translation initiation codon followed by 12–15 bases of the desired K_v1.6 sequence. Point mutations in the K_v1.6 N terminus were introduced by site-directed *in vitro* mutagenesis²¹. DNA sequences amplified by PCR were verified by sequencing²³.

Electrophysiology. CHO/dhfr[−] cells were microinjected¹² with solutions containing 50 ng μl^{−1} cDNA for α subunits with or without 450 ng μl^{−1} cDNA for β subunits and 20 ng μl^{−1} GFP-cDNA as a reporter gene. For whole-cell patch-clamp recordings, the pipette solution, and for inside-out recordings the bath solution, contained (in mM): 95 K-aspartate, 20 KCl, 1 CaCl₂, 1 MgCl₂, 11 EGTA, 10 HEPES, 2 glutathione, 2 Na₂ATP (pH 7.2 with KOH), 10 glucose and 20 sucrose (300 ± 10 mOsm). The bath solution (inside-out, pipette solution) contained (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂ and 5 HEPES (pH 7.4 with NaOH). Current recording, data acquisition, and analysis were as described^{6,12}. Shaker K_vα (ref. 13) and K_vβ1.1 (ref. 6) peptides (Research Genetics) dissolved in the bath solution were applied to inside-out patches by bath perfusion.

Immunoaffinity experiments. Rabbit polyclonal sera were raised against unique C-terminal sequences of K_v1.4 and K_v1.6 protein. The sequences of the synthetic peptides were SSL GDK SEY LEM EEG VKE SL (K_v1.4 residues 605–624)¹⁸ and RRS SYL PTP HRA YAE KRM (K_v1.6 residues 509–526)¹⁷. Anti-K_v1.4 and anti-K_v1.6 antibodies, purified and characterized essentially as described^{12,24}, were coupled to CNBr-activated Sepharose for immunoaffinity chromatography. Rat membrane protein solubilized in 2% Triton X-100 (ref. 24) was loaded onto the respective antibody column. Columns were washed with 30 bed volumes of 0.1% Triton X-100 in 20 mM Tris, 150 mM NaCl. Column-retained K_v1 channels were eluted with 0.1 M CAPS, pH 10, 0.1% Triton X-100, 150 mM NaCl. The eluate was cleared from leaching IgG by incubation with Protein A-Sepharose (30 min at 4°C) followed by dialysis against 0.1% SDS, 20 mM Tris/HCl, pH 7.4. Eluates were concentrated down to the original volume of the starting material (Amicon Centriprep) and separated by SDS–polyacrylamide gel electrophoresis under reducing conditions (1% β-mercaptoethanol). Western blotting was as described²⁴.

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Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils

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Nitric oxide (*NO) plays a central role in the pathogenesis of diverse inflammatory and infectious disorders^{1,2}. The toxicity of *NO is thought to be engendered, in part, by its reaction with superoxide (O₂^{•−}), yielding the potent oxidant peroxynitrite (ONOO[−])³. However, evidence for a role of ONOO[−] *in vivo* is based largely upon detection of 3-nitrotyrosine in injured tissues^{4–8}. We have recently demonstrated that nitrite (NO₂[−]), a major end-product of *NO metabolism, readily promotes tyrosine nitration through formation of nitryl chloride (NO₂Cl) and nitrogen dioxide (*NO₂) by reaction with the inflammatory mediators hypochlorous acid (HOCl) or myeloperoxidase^{9,10}. We now show that activated human polymorphonuclear neutrophils convert NO₂[−] into NO₂Cl and *NO₂ through myeloperoxidase-dependent pathways. Polymorphonuclear neutrophil-mediated nitration

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and chlorination of tyrosine residues or 4-hydroxyphenylacetic acid is enhanced by addition of NO_2^- or by fluxes of $^*\text{NO}$. Addition of $^{15}\text{NO}_2^-$ led to ^{15}N enrichment of nitrated phenolic substrates, confirming its role in polymorphonuclear neutrophil-mediated nitration reactions. Polymorphonuclear neutrophil-mediated inactivation of endothelial cell angiotensin-converting enzyme was exacerbated by NO_2^- , illustrating the physiological significance of these reaction pathways to cellular dysfunction. Our data reveal that NO_2^- may regulate inflammatory processes through oxidative mechanisms, perhaps by contributing to the tyrosine nitration and chlorination observed *in vivo*.

Monocytes and polymorphonuclear neutrophils (PMN) utilize the myeloperoxidase (MPO) $\text{H}_2\text{O}_2/\text{Cl}^-$ system to generate HOCl and other chlorinated oxidants as both a host defence mechanism and a means of invoking tissue injury^{11–14}. We have used human PMN to ascertain whether oxidation of NO_2^- by HOCl or MPO to form reactive nitrogen species has a physiological function. Because the oxidants formed by these reactions (NO_2Cl and $^*\text{NO}_2$) are reactive intermediates not directly detectable in cellular systems, 4-hydroxyphenylacetic acid (HPA) was used as a chemical probe to monitor nitration and chlorination reactions. The separation and

quantification of 3-chloro-HPA (Cl-HPA), 3-nitro-HPA (NO_2 -HPA) and 3,3'-bisHPA (Bi-HPA) was performed by high-performance liquid chromatography (HPLC) (Fig. 1a, d). PMN activated with 12-*D*-tetradecanoylphorbol-13-acetate (TPA, 50 ng ml^{-1}) released HOCl ($47.2 \pm 6.8 \mu\text{M}$) and yielded detectable levels of 3-chloro-HPA (Cl-HPA) ($0.8 \mu\text{M}$) after 60 min^{15} . Under these conditions, nitration of HPA was not observed (Fig. 1b, e) and NO_2^- was not detected in the incubation medium (not shown). In contrast, addition of NO_2^- to this reaction system at concentrations observed during inflammatory and infectious processes ($1\text{--}50 \mu\text{M}$; refs 16–18) increased Cl-HPA formation and induced nitration of HPA (Fig. 1c) to an extent dependent on the NO_2^- concentration (Fig. 1e). Nitrate (NO_3^-), however, had no effect on nitration and chlorination reactions. Product formation was maximal within 20–30 min of PMN stimulation and temporally paralleled superoxide ($\text{O}_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) production by PMN (data not shown), suggesting that these reactions are mediated by respiratory burst-derived oxygen metabolites¹¹. Qualitatively similar results were obtained when PMN adherent to fibronectin-coated cell culture wells were primed with tumour necrosis factor- α (TNF- α) (1 nM) and stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (100 nM). However, the yields of chlorinated and nitrated HPA were about fivefold less, a consequence directly related to decreased HOCl production ($9.2 \pm 2.1 \mu\text{M h}^{-1}$) and MPO release (not shown).

We observed that these actions of NO_2^- are relevant to nitration and chlorination of tyrosine residues (Fig. 1f). Incubation of three different tripeptides (Arg-Tyr-Gly, Gly-Tyr-Gly or Glu-Tyr-Gly) with stimulated PMN in the presence of NO_2^- led to the formation of 3-chlorotyrosine (Cl-Tyr) and 3-nitrotyrosine (NO_2 -Tyr) (Fig. 1f). In each case, the yields of Cl-Tyr exceeded those of NO_2 -Tyr, as observed for reactions of NO_2Cl (ref. 9). Because PMN are implicated in nearly all active inflammatory and immune processes, our observations suggest that these NO_2^- -dependent mechanisms may contribute to tyrosine nitration observed in such diverse pathologies as atherosclerosis⁴, rheumatoid arthritis⁵, septic lung injury⁶, inflammatory bowel disease⁷, and idiopathic pulmonary fibrosis⁸, a phenomenon previously attributed solely to ONOO $^-$.

To further elucidate mechanisms by which NO_2^- participates in PMN-mediated chlorination and nitration reactions, pathways involving reactive oxygen species production and MPO activity were modulated (Fig. 1g). Addition of Cu,Zn-superoxide dismutase (SOD) marginally enhanced, whereas catalase completely inhibited the formation of Cl-HPA and NO_2 -HPA, revealing a critical role for H_2O_2 . Inhibitors of MPO (sodium azide¹⁴ and aminobenzoic acid hydrazide (ABAH)¹⁹) completely abolished Cl-HPA and NO_2 -HPA formation, and caused >95% inhibition of Bi-HPA formation (not shown), a characteristic product of MPO-mediated reactions²⁰. When methionine was included to scavenge HOCl (ref. 15), Cl-HPA formation was completely abolished whereas NO_2 -HPA yields were only partially decreased (Fig. 1g). Residual nitration of HPA is attributed to direct reaction of NO_2^- with MPO compounds I and II forming $^*\text{NO}_2$ (ref. 10), which rapidly combines with phenoxyl radicals ($^*\text{HPA}$) generated by MPO²⁰ to form nitrated products (NO_2 -HPA), a process not noticeably affected by methionine²¹. Collectively, these data support a mechanism for PMN-mediated conversion of NO_2^- into nitrating and chlorinating intermediates that is dependent on active MPO.

A quantity of HOCl produced by stimulated PMN reacts with endogenously released taurine to form *N*-chlorotaurine¹⁴. Addition of NO_2^- before TPA stimulation resulted in a decrease in the yield of *N*-chlorotaurine (Fig. 2a), with a concomitant increase in Cl-HPA and NO_2 -HPA formation (Fig. 1f). In contrast, NO_2^- did not react with *N*-chlorotaurine preformed by activated PMN (Fig. 2a), illustrating that NO_2^- reacts with HOCl initially released from PMN to form NO_2Cl , but not with *N*-chlorotaurine over the incubation period. These observations were confirmed in cell-free,

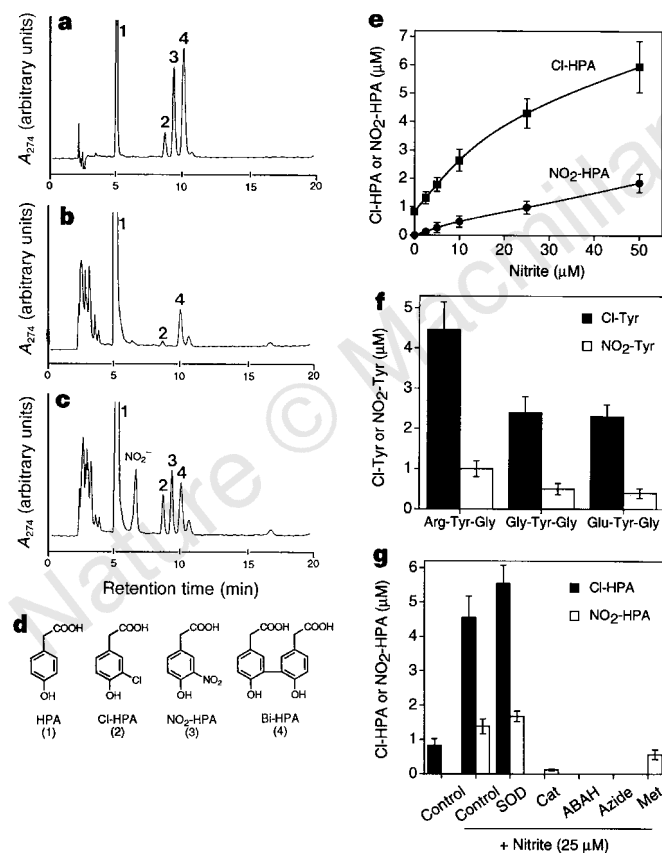


Figure 1 Nitrite enhances PMN-mediated chlorination and nitration of phenolic substrates by MPO-dependent mechanisms. HPLC separation of (a) standards of HPA (1), Cl-HPA (2), NO_2 -HPA (3) and Bi-HPA (4), and products formed following incubation of HPA with TPA-stimulated PMN in the absence (b) or presence (c) of NO_2^- ($50 \mu\text{M}$). d, Structures of HPA, Cl-HPA, NO_2 -HPA and Bi-HPA. e, Yields of Cl-HPA and NO_2 -HPA are dependent on the concentration of NO_2^- ($2\text{--}50 \mu\text{M}$). f, PMN-mediated chlorination and nitration of tyrosine residues in synthetic peptides in the presence of NO_2^- ($25 \mu\text{M}$). g, Effect of SOD (90 U ml^{-1}), catalase (500 U ml^{-1}), 4-aminobenzoic acid hydrazide (ABAH; $100 \mu\text{M}$), azide ($100 \mu\text{M}$) or methionine ($500 \mu\text{M}$) on PMN-mediated nitration and chlorination of HPA. In all experiments, unstimulated PMN did not catalyse formation of nitrated or chlorinated products, even in the presence of NO_2^- . Data represent the mean $\pm$ s.d. of at least four separate experiments.

chemical systems (Fig. 2b). The chlorinating activity of HOCl is greatly enhanced by conversion to more reactive species by catalytic anions at acidic pH (such as Cl_2 by Cl^- ; ref. 13). Analogously, we propose that NO_2^- converts HOCl into the more potent chlorinating species NO_2Cl at physiological pH. The strongly electron-withdrawing nature of the NO_2 group in NO_2Cl compared with the electron-donating properties of the OH group in HOCl enhances the Cl^+ -character of NO_2Cl and renders it a more potent agent of electrophilic aromatic chlorination. Subsequently, conversion of HOCl into NO_2Cl may divert the chlorinating reactivity to aromatic substrates such as tyrosine (Fig. 2c). Additionally, NO_2^- reacts with MPO compound II to regenerate the native ferric enzyme and $^*\text{NO}_2$ (Fig. 2c, ref. 10), itself a nitrating intermediate. Subsequent reac-

tions of $^*\text{NO}_2$ with biological substrates regenerates NO_2^- , thus serving a catalytic role in MPO-mediated reactions.

To simulate a physiological mechanism of NO_2^- formation (such as activated PMN vicinal to other $^*\text{NO}$ -producing cells), activated PMN were exposed to a continuous flux of $^*\text{NO}$, using the $^*\text{NO}$ -donor molecule PAPA NONate²². These conditions also promoted chlorination and nitration of HPA (Fig. 3a). Nitration of HPA was inhibited both by catalase and by azide at low fluxes of $^*\text{NO}$ (Fig. 3b), suggesting that under these conditions HPA nitration was dependent on H_2O_2 and MPO. At higher fluxes of $^*\text{NO}$, O_2^- is presumably consumed to form ONOO^- (ref. 3), decreasing the amount of H_2O_2 available as substrate for MPO and suppressing the production of MPO compound I and HOCl. Hence, under conditions of high $^*\text{NO}$

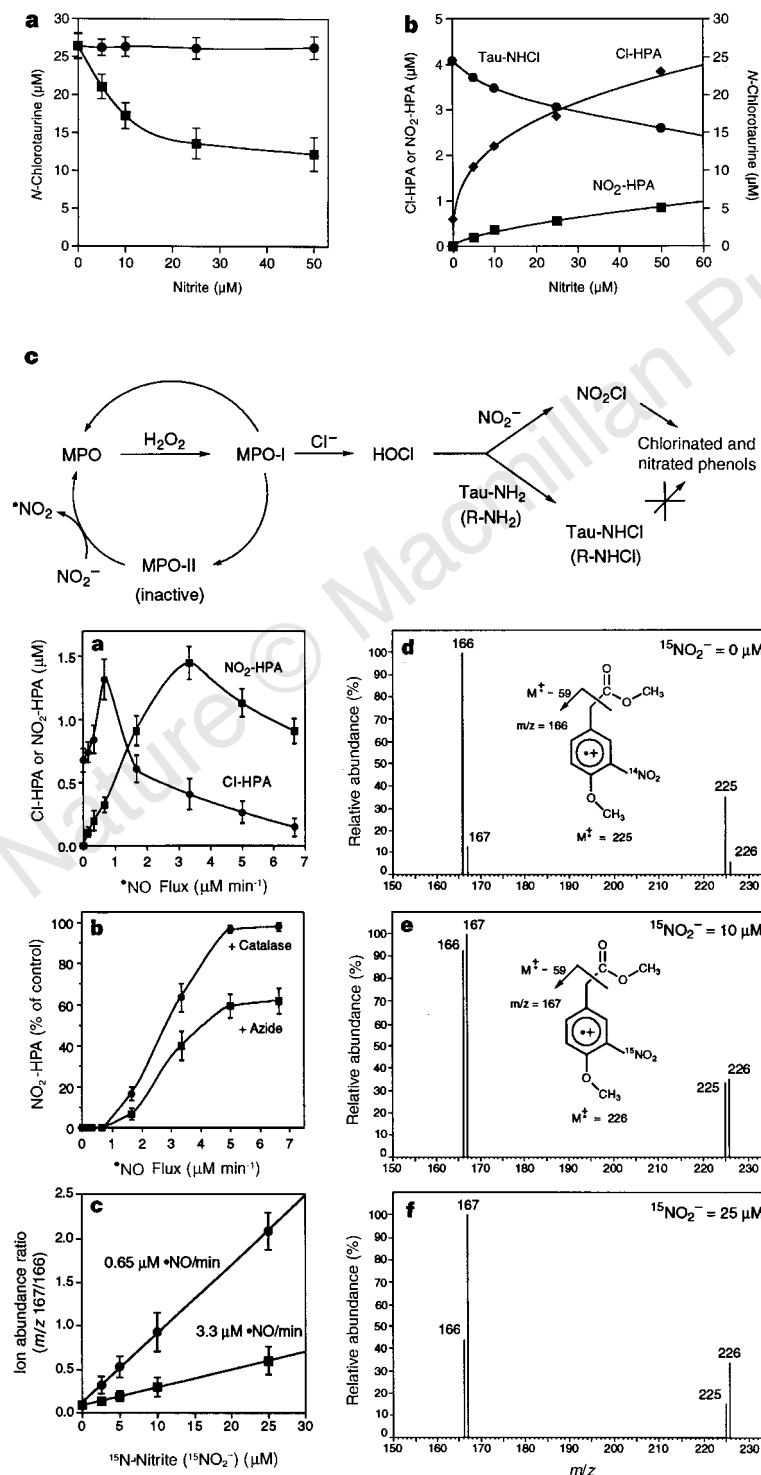


Figure 2 Nitrite competes with taurine for reaction with PMN-released HOCl. Treatment of PMN with TPA (50 ng ml⁻¹) caused release of *N*-chlorotaurine (26.5 ± 1.5 μM h⁻¹). **a**, Addition of NO_2^- to PMN before TPA treatment led to decreased *N*-chlorotaurine yield, especially at low NO_2^- concentrations (up to 10 μM) (squares). Addition of NO_2^- to *N*-chlorotaurine preformed by TPA-stimulated PMN did not decrease its concentration (circles). **b**, In cell-free reactions, effects of NO_2^- are analogous to those observed with PMN. Chemical reactions were initiated by bolus addition of reagent HOCl (40 μM) to continuously stirred solutions (1 ml) containing HPA (1 mM), taurine (40 μM) and NO_2^- (0–50 μM). Reactions were performed in the same buffer used for PMN experiments. The data are expressed as: **a**, the mean ± s.d. of four separate experiments, and **b**, the mean of three experiments. **c**, Schematic illustration of the pathways by which NO_2^- enhances PMN-mediated chlorination of phenolic substrates.

Figure 3 Dependence of NO_2^- on reaction pathways mediated by PMN exposed to $^*\text{NO}$. **a**, Formation of Cl-HPA and NO_2 -HPA by TPA-stimulated PMN in the presence of increasing rates of $^*\text{NO}$ release from PAPA NONate. **b**, Effect of catalase (500 U ml⁻¹) and azide (100 μM) on NO_2 -HPA formation by PMN exposed to $^*\text{NO}$. Heat-inactivated catalase displayed no inhibitory effect. **c**, Addition of increasing concentrations of $^{15}\text{NO}_2^-$ (2.5–25 μM) to stimulated PMN subjected to fluxes of $^*\text{NO}$ (0.65 or 3.3 μM min⁻¹) caused a linear increase in the ratio of fragment ion intensity ($m/z = 167/166$), a direct measure of the ratio $^{15}\text{NO}_2\text{-HPA}/^{14}\text{NO}_2\text{-HPA}$. Selected-ion monitoring mass spectrum of dimethylated $^{14}\text{NO}_2$ -HPA formed by TPA-stimulated PMN subjected to 0.65 μM $^*\text{NO}$ is illustrated in **d**. Addition of 10 μM (**e**) or 25 μM (**f**) $^{15}\text{NO}_2^-$ to the reaction mixture increased the relative abundance of ions $m/z = 226$ and 167, indicative of $^{15}\text{NO}_2$ -HPA formation (an increase of one mass unit due to ^{15}N). Ions formed at mass/charge ratios (m/z) of 225, 226 and 166, 167 represent the molecular ion and the major fragment for dimethylated $^{14}\text{NO}_2$ -HPA and $^{15}\text{NO}_2$ -HPA, respectively; electron impact ionization fragmentation is illustrated in the inset structures (**d** and **e**). Data are expressed as the mean ± s.d. from three separate experiments.

fluxes, catalase and azide less effectively inhibited HPA nitration, the former due partly to *NO -dependent catalase inhibition. Nitration of HPA by 3-morpholinysydnonimine (SIN-1, 1 mM), a species capable of simultaneously releasing *NO and O_2^- to generate $ONOO^-$, was not inhibited by catalase and only marginally affected (<15%) by azide (data not shown), further supporting the predominant role of MPO/NO_2^- rather than $ONOO^-$ in the nitration reactions we have observed.

To unequivocally demonstrate that NO_2^- is involved in phenolic nitration by activated PMN, we performed similar experiments in the presence of $^{15}NO_2^-$ and determined ^{15}N incorporation into NO_2 -HPA by gas chromatography/mass spectrometry (GC/MS). Increasing concentrations of $^{15}NO_2^-$ (2.5–25 μM) added to activated PMN exposed to two different rates of *NO production (0.65 $\mu M \text{ min}^{-1}$ or 3.3 $\mu M \text{ min}^{-1}$) resulted in a linear increase in the ratio of $^{15}NO_2$ -HPA/ $^{14}NO_2$ -HPA (Fig. 3c). Selected-ion monitoring mass spectrometry for the major fragment ion (m/z 166 and 167) for $^{14}NO_2$ -HPA and $^{15}NO_2$ -HPA, respectively, was used for quantification (fragmentation patterns illustrated in Fig. 3d, e). Mass spectra shown in Fig. 3d–f illustrate the increase in the relative abundance of the molecular ion and major fragment ion associated with $^{15}NO_2$ -HPA upon addition of increasing concentrations of $^{15}NO_2^-$ to PMN suspensions. Formation of $^{15}NO_2$ -HPA was completely abolished by catalase, azide and ABAH, and was largely inhibited by methionine (~70%) (not shown), confirming that extracellular NO_2^- is converted to nitrating species via MPO, and that this process competes with $ONOO^-$ -mediated pathways catalysed by human PMN.

Because inflammatory responses are causally linked to activation and adherence of PMN to target cells¹², we hypothesized that NO_2^- may critically influence PMN-mediated oxidative cellular injury. To test this possibility, activated PMN were co-cultured with bovine aortic endothelial cells (BAEC) in the absence or presence of NO_2^- . As previously shown²³, activated PMN induced a 40% decrease in the activity of BAEC angiotensin-converting enzyme (ACE) (Fig. 4). ACE activity was further decreased in a dose-dependent fashion by inclusion of NO_2^- to the cell system, suggesting that this may be an operative mechanism for hypotension associated with septic shock. The extracellular domain of ACE contains both an oxidant-sensitive zinc centre and a tyrosine critical for catalytic activity²⁴, suggesting that both sites may be important targets of reactive nitrogen species generated from NO_2^- . Although we have not directly shown that nitration and/or chlorination of the active-site tyrosine has occurred, this may be responsible for the observed inactivation of ACE and requires further investigation. These results suggest that the reactions we have described are relevant to inflammatory vascular injury, because (1) monocytes and macrophages and phagocytic cells implicated in the pathogenesis of diverse vascular diseases express MPO and produce HOCl upon stimulation^{11,12} and

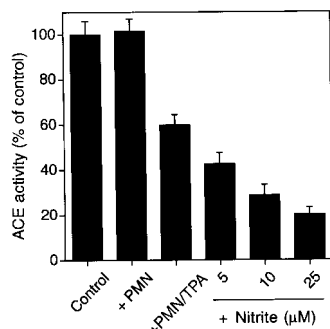


Figure 4 Nitrite exacerbates PMN-mediated inactivation of angiotensin-converting enzyme (ACE) in bovine aortic endothelial cells (BAEC). Addition of either unstimulated PMN (5×10^5 per well), TPA alone (5 ng ml^{-1}), or NO_2^- alone (25 μM) to BAEC monolayers had no effect on ACE activity. Data represent the mean $\pm$ s.d. of three separate experiments.

(2) tissues undergoing inflammatory responses contain elevated levels of MPO^{25} , $Cl\text{-Tyr}^{26}$ and $NO_2\text{-Tyr}^4$.

In summary, several lines of evidence show that activated human PMN convert NO_2^- into the inflammatory oxidants NO_2Cl and *NO_2 through MPO-dependent pathways. The data reveal that NO_2^- may play a role in phagocyte-mediated oxidative reactions at sites of inflammation and infection. Recent evidence illustrating colocalization of iNOS and MPO in PMN primary granules²⁷ infers that NO_2^- -dependent formation of NO_2Cl and *NO_2 in the phagolysosome may represent a previously unrecognized mechanism of host defence. This prospect is further strengthened by observations that (1) NO_2^- can enhance the bactericidal activity of MPO^{28} and (2) phagocytosed bacteria and model particles are nitrated²⁷ and chlorinated¹³ on tyrosine residues. In addition to serving as a 'footprint' of reactive nitrogen species, nitration of tyrosine (and presumably chlorination) could impact deleteriously on cellular function and viability because this specific modification is known to alter protein structure and function *in vitro* (for example, disassembly of structural proteins, inactivation of Mn-SOD, and inhibition of tyrosine phosphorylation; ref. 3). Although $NO_2\text{-Tyr}$ and $Cl\text{-Tyr}$ are highly elevated in various human diseases^{4–8,26}, the mechanisms of formation and the consequences of these protein modifications *in vivo* remain relatively unknown. Indeed, it has become evident that tyrosine nitration can be mediated by multiple pathways^{3,29,30} under different conditions, including the one we have described here, suggesting that $NO_2\text{-Tyr}$ should not be considered a specific marker of $ONOO^-$ formation, but as a collective indicator for the involvement of reactive nitrogen species. Our results illustrate new MPO-dependent pathways for formation of reactive nitrogen intermediates that may have broad implications for the regulation of local inflammatory and infectious events *in vivo*. □

Methods

Isolation and treatment of PMN. Human PMN were obtained from fresh venous blood from healthy individuals and isolated as previously described²⁰. Preparations contained >96% PMN, <2% eosinophils, and were >98% viable as assessed by trypan blue exclusion. PMN ($2 \times 10^6 \text{ ml}^{-1}$, unless otherwise indicated) were suspended in phosphate buffered saline (PBS) containing $MgCl_2$ (0.5 mM), $CaCl_2$ (1 mM) and glucose (1 mg ml^{-1}) to a final volume of 1 ml, and incubated with TPA (50 ng ml^{-1}) at 37°C with HPA (1 mM) or various peptides (1 mM; Stanford University Protein and Nucleic Acid Facility; >95% pure) in the presence or absence of NO_2^- . PMN were exposed to *NO by addition of (Z)-[N-(3-ammoniopropyl)-N-(n-propyl)amino]diazene-1-ium-1,2-diolate²² (PAPA NONOate, Alexis Corp.) (2.5–100 μM) 1 min following TPA addition. PAPA NONOate spontaneously releases 2 mol *NO per 1 mol donor molecule and has a $t_{1/2}$ of 15 min at 37°C and pH 7.4 (ref. 22). In some cases, $^{15}NO_2^-$ (Cambridge Isotope Laboratories, 98% ^{15}N) was preincubated with PMN for 5 min before stimulation with TPA and addition of PAPA NONOate. Reactions were terminated by placing tubes on ice and immediately adding SOD (90 U ml^{-1}), catalase (500 U ml^{-1}) and methionine (500 μM) to scavenge residual oxidants. When HOCl and N-chlorotaurine levels were determined, methionine was omitted. PMN were pelleted by centrifugation (1,200g, 10 min, 4°C) and the levels of products in the supernatant determined by HPLC.

High-performance liquid chromatography. HPA and its products were analysed by reverse-phase HPLC (5- μm Spherisorb ODS-2 column, 4 mm $\times$ 25 cm) using gradient elution (34% methanol to 40% methanol in 100 mM KH_2PO_4 , pH 3.0) over 25 min. Products were identified by UV detection (Waters 484) at 274 nm and were quantified by use of external standards. Peak identity was confirmed by retention time and by spectral match using photodiode array detection (Waters 996). Bi-HPA was specifically identified and quantified using an in-line fluorescence detector (Waters 470; λ_{ex} = 284 nm, λ_{em} = 410 nm)¹⁰. $Cl\text{-Tyr}$ and $NO_2\text{-Tyr}$ in peptides were determined by HPLC as previously described⁹.

Quantification of chlorinated oxidants. HOCl produced by activated PMN was determined by conversion of exogenously added taurine (10 mM, added before PMN activation) into stable N-chlorotaurine¹⁴. N-chlorotaurine in the

supernatant was then quantitatively determined by oxidation of 5-thio-2-nitrobenzoic acid (TNB) as previously described¹⁴. In the absence of added taurine, endogenously released *N*-chlorotaurine from activated PMN was determined in an identical manner.

¹⁵NO₂ incorporation. HPA and its reaction products in supernatants from pelleted PMN were extracted by vigorous shaking (10 min) with diethyl ether (3 × 5 ml). Organic extracts were combined and treated with a concentrated solution of ethereal diazomethane (CH₂N₂) to methylate the phenolic and carboxyl functional groups. Extracts were dried over anhydrous Na₂SO₄ (60 min, 4°C) and concentrated to dryness by a stream of N₂. Residues were resuspended in ethyl acetate (100 µl) and injected (splitless mode, 2 µl) on a Hewlett-Packard gas chromatograph (Model HP 6890) equipped with a DB-5 fused-silica capillary column (12.5 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific) interfaced to a mass selective detector (MSD, HP Model 5972). Column temperature was increased from 100°C to 170°C at 8°C min⁻¹. Selected-ion monitoring (SIM) (*m/z* 166, 167, 225 and 226) was performed using electron ionization at 70 eV with the ion source maintained at 190°C. For quantification, SIM spectra were background-subtracted and the ratios of ion intensities determined. Peak identity was confirmed by matching retention time and MS spectra with derivatized NO₂-HPA standard. CH₂N₂ was synthesized using a Diazald kit according to the manufacturer's instructions (Aldrich Chemical Co.).

Endothelial cell/PMN cultures. Confluent bovine aortic endothelial cells (BAEC, passages 5–7) were cocultured with isolated human PMN (5 × 10⁵) in 24-well plates (1 ml Earle's salt solution, ESS) as previously described²³. Cocultures were treated with TPA (5 ng ml⁻¹) in the presence or absence of NO₂⁻ (5, 10 or 25 µM) for 1.5 h at 37°C in humidified 5% CO₂. In control experiments, BAEC were treated with either TPA alone, unstimulated PMN or NO₂⁻ alone. Following incubation, monolayers were gently washed with ESS (3 × 1 ml) and incubated for an additional 30 min with the ACE substrate [³H]benzoyl-Phe-Ala-Pro ([³H]BPAP, 0.1 µCi ml⁻¹; 22 Ci mmol⁻¹). ACE activity in BAEC was determined following extraction as previously described²³.

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HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes

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Cytotoxic T lymphocytes (CTLs) lyse virally infected cells that display viral peptide epitopes in association with major histocompatibility complex (MHC) class I molecules on the cell surface. However, despite a strong CTL response directed against viral epitopes, untreated people infected with the human immunodeficiency virus (HIV-1) develop AIDS. To resolve this enigma, we have examined the ability of CTLs to recognize and kill infected primary T lymphocytes. We found that CTLs inefficiently lysed primary cells infected with HIV-1 if the viral *nef* gene product was expressed. Resistance of infected cells to CTL killing correlated with *nef*-mediated downregulation of MHC class I (ref. 1) and could be overcome by adding an excess of the relevant HIV-1 epitope as soluble peptide. Thus, Nef protected infected cells by reducing the epitope density on their surface. This effect of *nef* may allow evasion of CTL lysis by HIV-1-infected cells.

It has been difficult to demonstrate directly that CTLs efficiently recognize and lyse HIV-1-infected primary lymphocytes, in part because of the problem of working with heterogeneous cell populations consisting of both infected and uninfected cells. We examined the efficacy of CTL killing by using a flow-cytometric CTL killing assay which identified infected cells marked with placental alkaline phosphatase (PLAP). We found that primary T lymphocytes

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infected with an HIV-1 encoding PLAP but lacking multiple accessory factors, including *nef*, (HXB-PL nef^{-} ; Fig. 1a), were susceptible to CTL killing. The light-scattering plot of untreated virus-exposed cells indicated a fairly uniform oval-shaped population of cells which was electronically gated (lymphocyte gate in Fig. 2, panel 1). On the basis of the expression of PLAP, 28% of these cells were determined to be HIV-infected (Fig. 2, panel 2). Following treatment with an HIV-1 *gag*-specific CTL clone, the percentage of PLAP $^{+}$ cells fell to 6% of the cells in the lymphocyte gate (Fig. 2, panel 5) and a new population of small PLAP $^{+}$ cells appeared outside this gate (Fig. 2; compare panels 3 and 6). That the cells remaining in the lymphocyte gate were alive was supported by the fact that they did not take up a fluorescent dye (7-aminoactinomycin D, 7AAD) specific for dead cells^{2,3}. In contrast, the newly appearing small cells did take up 7AAD (data not shown). Thus, flow cytometry allowed us to identify infected cells that had survived CTL treatment.

To investigate whether expression of the accessory gene *nef* influenced CTL recognition, we infected primary cells with a *nef* $^{+}$ molecular clone (HXB-PL nef^{+} ; Fig. 1a). We observed a dramatic reduction in the level of surface MHC class I HLA-A2 (Fig. 3a, panel 1) with infection and found that these cells were virtually resistant to the HLA-A2-restricted anti-HIV-*gag* CTL clone 18030D23 (ref. 4) (Fig. 3a; compare panels 1 and 4; see also Fig. 3b). In contrast, cells infected with a virus lacking a functional *nef* gene did not downregulate MHC class I and ~90% of the cells were killed (Fig. 3a; compare panels 2 and 5; see also Fig. 3b). The observed killing was appropriately HLA-restricted, because cells lacking HLA-A2 and infected with HXB-PL nef^{-} were not lysed by the HLA-A2-restricted CTL clone (Fig. 3a; compare panels 3 and 6; see also Fig. 3b). To ensure that small differences in infection rate did not affect our results, we used very large effector:target ratios. For example, if only the infected cells were considered as true targets, when 12% of the cells were infected the maximal effector:true target ratio was 42:1 (Fig. 3b).

HIV-infected cells expressing *nef* were also resistant to lysis by the anti-*gag* CTL clone 161JXA14. This clone recognizes the same *gag* epitope as 18030D23, but was isolated from a different HIV-1-infected patient (see Methods). In this experiment, some cells infected with a *nef* $^{+}$ virus had not fully downregulated MHC class I, and only about 30% of these cells survived, compared with ~70% survival of those cells that had fully downregulated MHC class I (Fig. 4a, b). *Nef* $^{-}$ infected cells (which did not downregulate MHC

class I at all) were once again efficiently killed (Fig. 4a, compare panels 1 and 3; see also Fig. 4b). To confirm that cells surviving CTL treatment were alive, CTL-treated cells were analysed in duplicate with or without 7AAD staining. The percentage of cells surviving was the same whether living cells were identified by light-scattering characteristics alone or by light-scattering characteristics plus 7AAD exclusion (Fig. 4c).

The anti-RT CTL clone 68A62 (refs 5, 6) killed *nef* $^{-}$ cells less efficiently than the anti-*gag* CTL clones (about 50% lysis), consistent with the reduced level of reverse transcriptase epitope found on the surface of infected cells^{7,8}. Only ~20% of *Nef*-expressing cells were killed following 68A62 treatment (data not shown), demonstrating that all clones tested had difficulty killing cells infected with a *nef* $^{+}$ virus.

To express both PLAP and *nef* in the HIV-1 constructs described above, an internal ribosome entry site (IRES^{9,10}) had been introduced 5' to the *nef* gene, which results in *nef* overexpression¹¹ (Fig. 1a). We therefore tested the HXB-EP construct¹¹, which expresses *nef* under the control of its usual regulatory elements (Fig. 1b). We found that HXB-EP-infected cells also downregulated MHC class I (up to 300-fold; data not shown) and survived treatment with anti-HIV CTL (clone 161JXA14) 24-fold better than control cells infected with HXB-EP nef^{-} (Table 1).

Protection of HIV-infected cells correlated with downregulation of MHC class I and therefore appeared to result from a decreased epitope density on the infected cell surface. To confirm that this was the mechanism, infected target cells were sensitized to CTL killing by incubation with the relevant HLA-A2-restricted *gag* peptide epitope^{5,7,8}. Peptide pulsing did not alter *nef*-mediated MHC class I downregulation (data not shown), but it did result in killing of cells infected with a *nef* $^{+}$ virus (Fig. 4d). In this experiment, only the infected subset of non-peptide treated cells were true targets (in the *nef* $^{+}$ sample, 12%). By comparison, most, perhaps all, of the peptide-pulsed cells were true targets. Thus, effective killing of the *nef* $^{+}$ peptide-pulsed cells occurred at an effector:true target ratio eightfold lower than that to which untreated *nef* $^{+}$ cells were resistant. This experiment demonstrates that a low level of HLA-A2 present on cells infected with *nef* $^{+}$ virus was available to exogenous soluble peptide. Once recognized, infected cells could be killed, indicating that resistance to CTL killing occurred at the level of epitope presentation.

The HXB2D-derived viruses described here do not have functional alleles for the viral accessory proteins *vpu* and *vpr* (Fig. 1). We

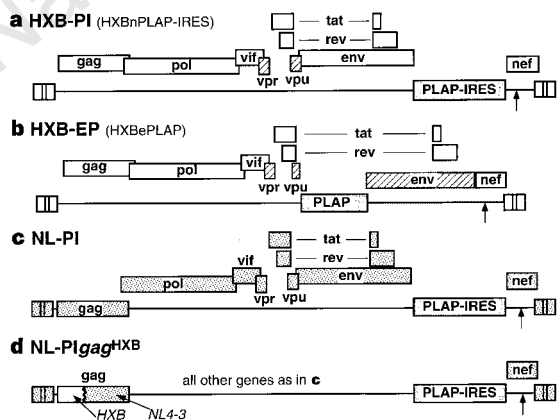


Figure 1 Genomic organization of HIV-1 vectors containing PLAP. **a**, HXB-PI and **b**, HXB-EP are derived from the molecular clone HXB2D; **c**, **d**, NL-PI vectors are derived from the molecular clone NL4-3 and have open reading frames for all accessory proteins. Viral genes that are hatched are not expressed. Expressed HXB genes are open boxes and expressed NL4-3 genes are stippled boxes. Vertical arrows indicate positions of the mutation in the *nef* $^{-}$ variant: an *Xho*I site was destroyed to abolish *Nef* function. Vector names in parentheses are designated according to ref. 11.

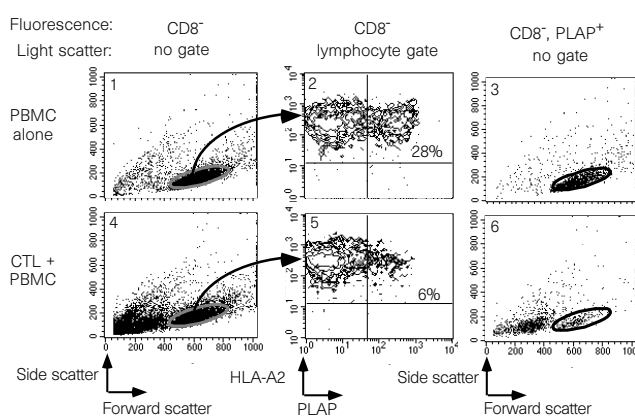


Figure 2 Flow-cytometric measurement of anti-HIV CTL-mediated cytotoxicity (clone 161JXA14). Target cells were mock-treated (panels 1–3) or CTL-treated at an effector:target ratio of 5:1 (panels 4–6). The grey circle (lymphocyte gate) indicates the population of cells analysed to generate the fluorescence plots (panels 2 and 5). Uninfected control cells were all PLAP $^{-}$ and fell to the left of the vertical line. Panels 3 and 6 are the light-scatter plots of all PLAP $^{+}$, CD8 $^{+}$ cells.

therefore inserted PLAP into another HIV-1 molecular clone, NL4-3 (NL-PI; Fig. 1c and d), that expresses these genes (and contains many other variations in sequence). In NL-PI there are two amino-acid changes within the relevant *gag* epitope which result in non-recognition by the anti-*gag* CTL clones described above (S.A.K., manuscript in preparation). Therefore to create a version that could be recognized by the CTL clones, the amino terminus of HXB-PI *gag* was inserted into NL-PI (NL-PI^{HXB}; Fig. 1d).

The results we obtained with NL-PI^{HXB} were very similar to those we obtained with HXB-PI. Cells infected with NL-PI depended on *nef* for MHC class I downregulation; there was no more than a twofold residual downregulation in the absence of a functional *nef* gene (Fig. 5a, panel 1). Downregulation of MHC class I by Nef protected cells from CTLs: none of these cells were killed (Fig. 5b). A role for other protective factor(s) in NL-PI was

suggested by the fact that *nef*⁻ NL-PI constructs survived better than *nef*⁻ HXB constructs (40% versus 10% survival respectively; Fig. 5b).

The epitope specificity of our assay was demonstrated by the fact that cells infected with NL-PI^{HXB} (unrecognized *gag* epitope; see Methods) were inefficiently recognized and killed (6% lysis), whereas 60% of cells infected with NL-PI^{HXB} *nef*⁻ (cognate epitope) were killed (Fig. 5b).

So far we have demonstrated Nef-mediated MHC class I downregulation and protection from CTLs in primary CD4⁺ lymphocytes from eight different donors. The observed protection ranges from 2- to 34-fold depending on the level of cytotoxicity of the CTLs. We noted a greater difference between *nef*⁺ and *nef*⁻ target-cell killing when the CTLs were more active because a higher proportion of *nef*⁻ cells were killed. We expect that the observed protection

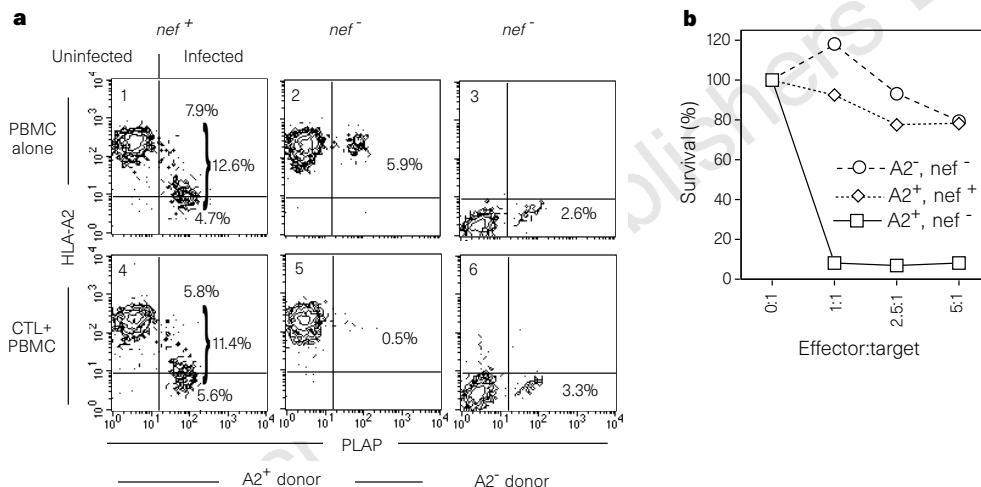


Figure 3 *Nef* protects cells from killing by the *gag*-specific CTL clone 18030D23. **a**, Cytofluorometric CTL killing assay. Panels 1-3 are plots of mock-treated infected PBMCs expressing *nef* as indicated (one of two duplicates is shown). Panels 4-6 are plots of CTL-treated at an effector to total target ratio of 1:1. Percent

PLAP⁺ is indicated for each panel. Uninfected control cells were all PLAP⁻ and fell to the left of the vertical line. HLA-A2 negative cells fell below the horizontal line. **b**, Graphical representation of the experiment described in **a**, showing the effects of increasing effector:target ratios.

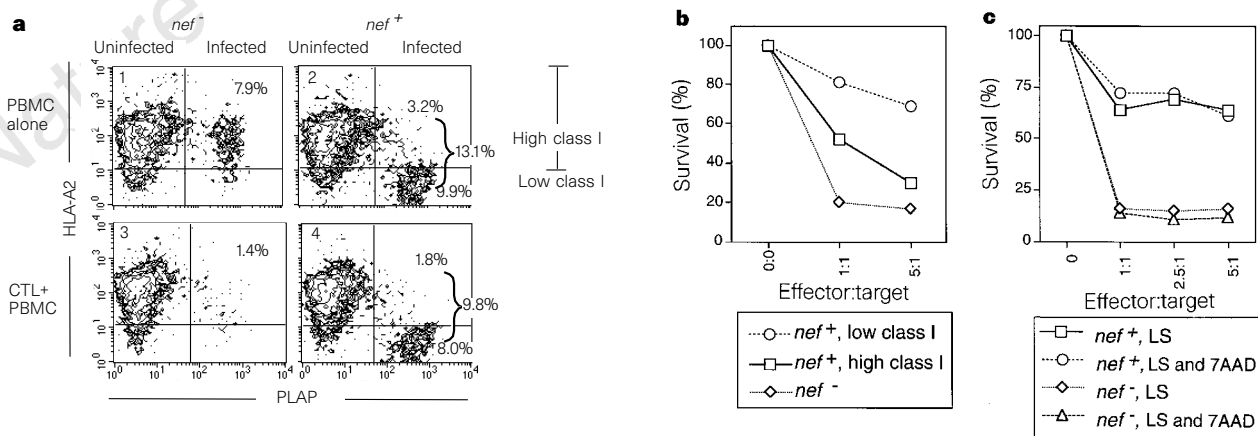


Figure 4 *Nef* protects cells from killing by CTL clone 161JXA14. **a**, Cytofluorometric killing assay of PBMCs treated with anti-HIV-*gag* CTL clone 161JXA14. Panels 1 and 2 are mock-treated PBMCs (one of two duplicates is shown). Panels 3 and 4 are CTL-treated at an effector to total target ratio of 1:1. Uninfected control cells were all PLAP⁻ and fell to the left of the vertical line. HLA-A2-negative cells fell below the horizontal line. Percentage PLAP⁺ is indicated in each panel. **b**, Graphical representation of the CTL assay described in **a**. **c**, 7AAD staining of CTL-treated cells. Surviving cells were those with characteristic light scatter (LS) staining negative for 7AAD (see Methods). Before CTL treatment, all cell samples were 8% PLAP⁺. **d**, CTL killing of peptide-pulsed targets. Target cells described in Fig. 2 legend were treated with soluble *gag* peptide epitope (amino acids 77-85, SLYNTVATL) and treated with CTL clone 18030D23.

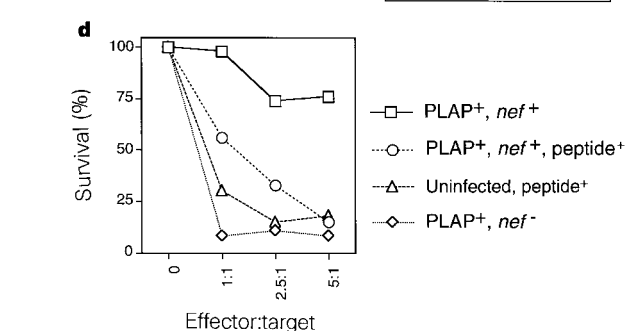


Table 1 Protection of PBMCs infected with HXB-EP containing an intact *nef* gene against CTL lysis

Genotype		Per cent PLAP ⁺		Per cent surviving
		-CTL	+CTL	
HXB-PI ^{nef} +	Low class I	8.3	5.3	64
	High class I	4.1	1.0	24
HXB-PI ^{nef} -	Low class I	8.7	0.5	6
	High class I	9.0	4.4	49
HXB-EP ^{nef} +	Low class I	2.8	0.5	18
	High class I	14.4	0.3	2

Results shown are for an effector:target ratio of 1:10.

from HLA-A2-restricted CTLs will extend to other HLA-restricted CTLs because we have also observed downregulation of HLA-A3, B7, B51 and one C allele (data not shown). Because all of these experiments were done with infected cells, it is not clear whether *nef* is sufficient for protection from CTLs. Although the protective effect of *nef* in this assay was due to a reduction of epitope density on the cell surface, other mechanisms of CTL evasion, such as *nef*-mediated upregulation of Fas ligand¹² and the generation of HIV sequence variants^{13–15}, may also play a role *in vivo*.

A partial reduction in surface MHC class I with HIV-1 infection has been reported previously^{8,16,17} and has been attributed to *nef*, *vpu* or *tat*^{1,18,19}. We found that *nef* had a profound (up to 300-fold) effect on surface class I in primary cells and had functional significance because *nef* protected infected cells from anti-HIV CTL recognition. Although these results help explain how HIV evades immune surveillance, they raise the question of how anti-HIV CTLs are generated *in vivo*. One possibility is that the epitope density on infected cells is sufficient for CTL activation but not for lysis, as has been observed for Epstein–Barr virus²⁰. Alternatively, HIV epitopes may be presented in association with MHC class I by uninfected antigen-presenting cells (for review, see ref. 21). These results also

raise the question of whether class I downregulation renders infected cells susceptible to lysis by natural killer cells *in vivo*.

Different *nef* alleles may vary in their ability to downregulate class I, and in an individual this may help determine how effectively CTLs can combat HIV-1. A strong anti-HIV CTL response could generate a lower 'set point' of steady-state viraemia, reached after resolution of the acute infection and slow disease progression^{22–24}. Therapeutic agents that inhibit the action of *nef* might then shift the balance in favour of the host's CTLs and improve the outcome of individuals infected with HIV-1.

Methods

HIV-1 constructs. A description of the HXB-derived vectors is published elsewhere¹¹. The NL-PI vectors were derived from the NL4-3 molecular clone²⁵ (from M. Martin through the AIDS Reference and Reagent Repository). PLAP was inserted after using polymerase chain reaction (PCR) to amplify a fragment containing the PLAP gene and the IRES from HXBnPLAPIRES^{nef}¹¹; the following primers were used: 5'-ATTAGTGAACAGATCTTTGGCACT-TATCTGGGA-3' and 5'-GTGAATTAGCCCTTCCAGTCCC-3'. The resultant fragment was digested with *Bgl*II and *Xho*I and ligated into the unique *Bam*HI and *Xho*I sites of NL4-3 to create NL-PI^{nef}+. The HLA-A2-restricted anti-gag CTL clones recognize HXB gag amino acids 77–85 (SLYNTVATL), but do not recognize the NL4-3 gag epitope (SLYNTIAVL; S.A.K., manuscript in preparation). To create a chimaeric virus that would be recognized, the *Bss*HII–*Spe*I fragment encoding the N-terminal 221 amino acids of gag in HXB-PI was cloned into NL-PI to create NL-PI^{gag}HXB. All *nef* mutants had N-terminal frameshift mutations constructed by filling in the unique *Xho*I site. To prepare homogeneous viral preps, virus was not propagated in tissue culture, but was generated directly from transfected 293 cells as described¹¹. Experiments using HXB-EP required pseudotyping as described¹¹, except that an HXB2 envelope expression vector was used.

Cell culture. HIV-seronegative peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the Massachusetts General Hospital blood bank. PBMCs were further purified by Ficoll–Hypaque centrifugation. Non-adherent cells were depleted for CD8⁺ cells with magnetic beads (Dynabeads). The remaining cells were stimulated with phytohaemagglutinin (PHA; Sigma) at 5 µg ml⁻¹. Twenty-four hours post-stimulation, interleukin (IL)-2 (Genzyme) was added at 50 units ml⁻¹. At 2–6 days post-stimulation, one million fresh PBMCs, or PBMCs carried in culture for less than four weeks, were infected by co-culture or spin infection (1 ml viral supernatant in 4 µg ml⁻¹ polybrene 1.5 h at 2,500 r.p.m. in a table-top centrifuge)²⁶. (Spin infection gave more reproducible results than co-culture.) We have found that lymphoblasts remained infectable in culture for about four weeks with weekly restimulation by PHA and irradiated feeder cells.

CTL clones. CTL clones were isolated by limiting dilution from HIV-1-infected individuals. Clonality of the line was established by demonstration of unique T cell receptor usage (data not shown). The CTL clones were maintained in culture with periodic re-stimulation as previously described^{15,6}. CTL clones 18030D23 and 161JXA14 both recognize HIV gag amino acids 77–85; SLYNTVATL (ref. 4 and S.A.K., unpublished data). CTL clone 68A62 recognizes reverse transcriptase amino acids 476–484 (refs 5–7, 27).

CTL assay. At 2–6 days after infection, 100,000 infected target cells were mixed with the indicated ratio of effectors in a microtitre-well plate, centrifuged for 3 min at 1,000 r.p.m. and incubated for 4 h at 37 °C. Cells were then stained with polyclonal antibody directed against PLAP (DAKO), a monoclonal antibody directed against HLA-A2 (One Lambda) and, in some cases, a Cy-chrome-conjugated antibody against CD8 (Pharmingen). Secondary antibodies were either a combination of goat anti-mouse immunoglobulin (IgG) fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG phycoerythrin (PE), or streptavidin-PE and goat anti-rabbit-FITC. Infected cells were then fixed with 2% paraformaldehyde in PBS overnight at 4 °C.

Cytofluorimetry was done on a Becton Dickinson FACSCAN and analysed with Cell Quest software. If three colour staining was used, data from 5,000 CD8⁺ cells with the proper light-scattering characteristics were collected. If 7AAD was added with the secondary antibodies, both CD8–Cy-chrome and 7AAD-staining (dead) cells were omitted from the analysis on the basis of a high fluorescence signal in the FL-3 channel. When an antibody against CD8

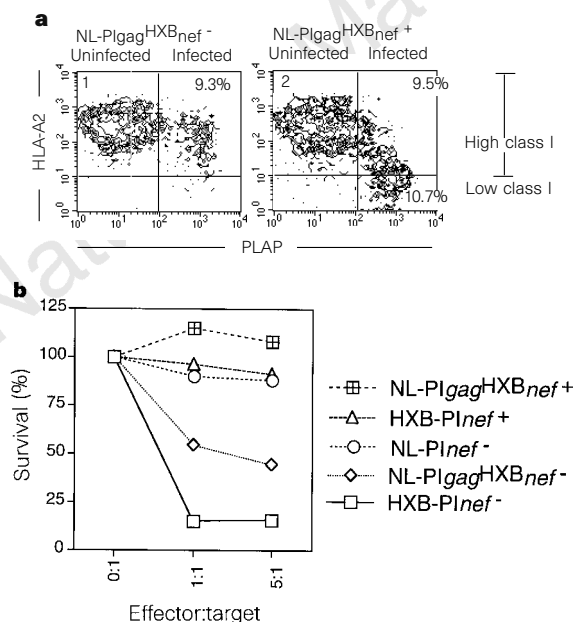


Figure 5 Cells infected with the NL-PI HIV-1 molecular clone are protected by Nef against CTL lysis, and CTL killing detected by cytofluorimetry is dependent on expression of the appropriate epitope. **a**, Downregulation of class I in PBMCs infected with NL4-3-derived constructs plus and minus *nef* expression. Percentage PLAP⁺ for each sample is as indicated. HLA-A2-negative cells fell just below the horizontal line and uninfected cells fell to the left of the vertical line in each panel. **b**, Cytofluorimetric killing assay of cells infected with the indicated viral constructs. For *nef*⁺ samples, only PLAP⁺ cells with low levels of class I were considered. 26% of HXB-PI^{nef}+, 39.6% of HXB-PI^{nef}+, 12.6% of NL-PI^{nef}- were infected. Infection rates of NL-PIgagHXB^{nef} constructs are shown in **a**.

was not used (Fig. 3), target cells were gated away from CTLs by light-scatter properties: CTLs had significantly more side scatter than PBMCs. The number of total cells collected per sample was adjusted for the addition of CTLs such that data for 10,000 target cells were collected. The percentage of alive PLAP⁺ cells was used to calculate per cent survival as follows: ((percentage PLAP⁺ in CTL-treated sample) ÷ (the average percentage PLAP⁺ of two duplicates to which no CTLs were added)).

Peptide pulsing. One million target cells were incubated with the HLA-A2-restricted gag peptide epitope (amino acids 77–85, SLYNTVATL) at 100 µg ml⁻¹ for 90 min at 37 °C under 5% CO₂. Pulsed cells were washed twice with RPMI plus 5% fetal calf serum. Because both uninfected and infected cells were targets, per cent survival was determined by the number (not the percentage) of cells remaining ((number of CTL-treated target cells remaining (PLAP-positive or negative as indicated)) ÷ (the average number of cells (PLAP positive or negative as indicated) in two duplicate mock-treated samples)).

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Synergistic actions of Rad51 and Rad52 in recombination and DNA repair

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In the yeast *Saccharomyces cerevisiae*, mutations in the genes *RAD51* or *RAD52* result in severe defects in genetic recombination and the repair of double-strand DNA breaks. These genes, and others of the *RAD52* epistasis group (*RAD50*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11* and *XRS2*), were first identified by their sensitivity to X-rays¹. They were subsequently shown to be required for spontaneous and induced mitotic recombination, meiotic recombination, and mating-type switching (reviewed in ref. 2). Human homologues of *RAD50*, *RAD51*, *RAD52*, *RAD54* and *MRE11* have been identified^{3–6}. Targeted disruption of the murine *RAD51* gene results in an embryonic lethal phenotype, indicating that Rad51 protein is required during cell proliferation^{7,8}. Biochemical studies have shown that human *RAD51* encodes a protein of relative molecular mass 36,966 (hRad51) that promotes ATP-dependent homologous pairing and DNA strand exchange^{9–11}. As a structural and functional homologue of the RecA protein from *Escherichia coli*^{3,9,12}, hRad51 is thought to play a central role in recombination. Yeast Rad51 has been shown to interact with Rad52 protein^{13–15}, as does the human homologue¹⁶. Here we show that hRad52 stimulates homologous pairing by hRad51. The DNA-binding properties of hRad52 indicate that Rad52 is involved in an early stage of Rad51-mediated recombination.

To facilitate the preparation of human Rad52 protein (hRad52), the *RAD52* gene was amplified by the polymerase chain reaction (PCR) from plasmid pCR52 carrying cDNA previously isolated from a human testis cDNA library (provided by O. Bezzubova and J.-M. Buerstedde) and cloned into the bacterial expression vector pET15b using flanking primers (Fig. 1a). The sequence of the cloned human *RAD52* gene was confirmed using an automated DNA sequencer. Recombinant hRad52 protein (carrying an amino-terminal six-histidine tag) was overexpressed from the resulting plasmid (pFB581; Fig. 1b), and purified to homogeneity as shown in Fig. 1c, d.

In *S. cerevisiae*, *rad51* and *rad52* mutants accumulate, but fail to process, double-strand breaks in DNA, indicating that the Rad51 and Rad52 proteins are directly involved in the formation of heteroduplex DNA¹⁵. Because human Rad51 and Rad52 interact¹⁶, we investigated the actions of hRad52 in hRad51-mediated homologous pairing assays. It was previously shown that hRad51 binds both single- and double-stranded DNA to form helical nucleoprotein filaments similar to those made by *E. coli* RecA protein¹². In the presence of ATP, hRad51 single-stranded DNA (ssDNA) filaments interact with homologous duplex DNA to form joint molecules⁹. The reactions are stimulated by replication protein A (RPA), a single-stranded DNA-binding protein that helps to remove secondary structure from the ssDNA¹⁰.

Efficient joint molecule formation by hRad51 occurs *in vitro* over a very narrow concentration range, with maximal efficiency observed at a ratio of one hRad51 monomer per three nucleotides of ssDNA⁹. This binding ratio corresponds to the amount of hRad51 required to fully saturate the ssDNA¹², suggesting that homologous pairing occurs only when the ssDNA is fully coated with a hRad51 filament. Incubation of single-stranded circular DNA with subsaturating

amounts of hRad51 (one hRad51 per six nucleotides), followed by the addition of ^{32}P -labelled linear duplex DNA, results in the formation of few, if any, joint molecules (Fig. 2, lane 2). However, the presence of hRad52 led to a significant stimulation in the yield of joint molecules formed by hRad51 (Fig. 2, lane 3). Joint molecule formation was dependent on DNA homology (data not shown). In the absence of hRad51, hRad52 alone was unable to promote joint molecule formation (Fig. 2, lane 4). Stimulation of hRad51 by hRad52 was also observed in the presence of RPA.

To understand how hRad52 stimulates hRad51, we analysed the DNA-binding properties of hRad52 using oligonucleotides (62 nucleotides long). hRad52 bound ssDNA, as determined by polyacrylamide gel electrophoresis (Fig. 3a, lanes 2–8) to form large protein–ssDNA complexes that failed to enter the gel. When the oligonucleotide was annealed with its complementary strand to form duplex DNA, a reduced level of binding was observed (Fig. 3a, lanes 9–15). At higher protein concentrations, however, binding to duplex DNA was observed (data not shown).

Owing to the difficulty in interpreting complexes that fail to enter polyacrylamide gels, the hRad52–ssDNA complexes were analysed by gel electrophoresis through 0.8% agarose. Discrete protein–DNA complexes were now observed (Fig. 3b, lanes 2–6), and a distinct optimum for DNA binding was seen at low (0.5–1.0 mM) Mg^{2+} concentrations (Fig. 3c, lanes 2 and 3). Under these conditions, 100% of the ssDNA (100 nM) was bound by 48 nM hRad52. The observed Mg^{2+} optimum corresponds to the requirement of hRad51 for divalent metal ions in joint molecule formation¹⁰. Mn^{2+} , Ca^{2+} and, to a lesser extent, Zn^{2+} could substitute for Mg^{2+} for ssDNA binding by hRad52 (data not shown). hRad52–ssDNA complexes formed efficiently in 0.3 M KCl, and binding was neither stimulated nor reduced by the presence of ATP (data not shown). These results show that hRad52 binds short single strands rather than duplex DNA to form stable complexes. It was previously shown that the Rad52 protein can promote the reannealing of two complementary DNA strands^{17,18}.

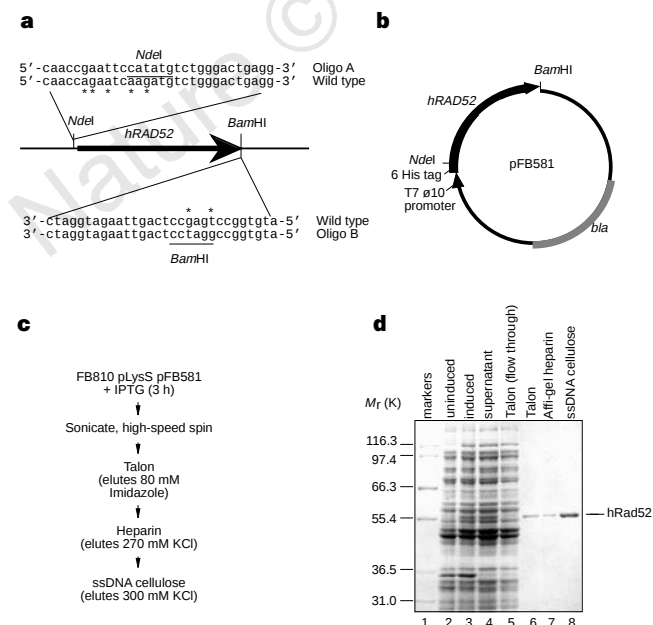


Figure 1 Purification of recombinant human Rad52 protein from *E. coli*. The *RAD52* gene was cloned into pET15b using two flanking primers (**a**) to produce plasmid pFB581 (**b**). The *hRAD52* and β -lactamase (*bla*) genes are indicated. His-Rad52 was overexpressed in the presence of IPTG and purified by three chromatographic steps (**c**). The overexpression and purification of recombinant hRad52 protein, analysed by 10% SDS-PAGE, is indicated in (**d**). Proteins were visualized by staining with Coomassie blue.

Because hRad52 binds ssDNA and interacts specifically with hRad51 protein, we reasoned that the stimulatory effect of hRad52 on hRad51-mediated homologous pairing reactions may result from hRad52 facilitating the interaction of hRad51 with the ssDNA substrate. To test this hypothesis, homologous pairing reactions were set up in which the order of protein addition was varied. In this experiment, we again used a limiting amount of

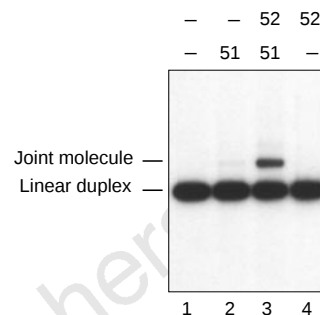


Figure 2 Stimulation of hRad51-mediated joint molecule formation by hRad52. Single-stranded circular DNA (4.3 kilobases) was incubated with hRad52 (52; 0.8 μM) and/or hRad51 (51; 5 μM) as indicated for 5 min at 37°C. Homologous 5' ^{32}P -labelled linear duplex DNA was then added and incubation was continued for a further 2 h at 37°C. The resulting joint molecules were stabilized and deproteinized. ^{32}P -labelled DNA was analysed by agarose gel electrophoresis followed by autoradiography.

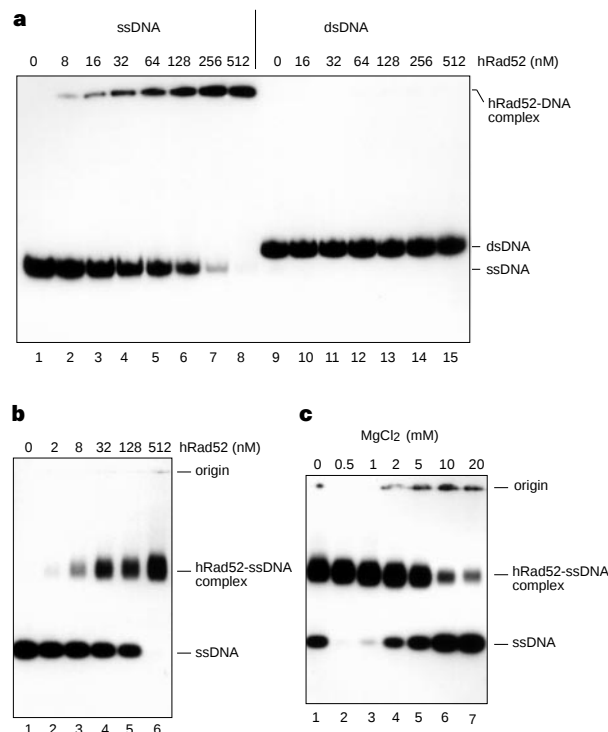


Figure 3 DNA-binding properties of hRad52. **a**, The indicated concentrations of hRad52 were incubated with 100 nM ^{32}P -labelled single-stranded (ss; lanes 1–8) or 200 nM duplex DNA (ds; lanes 9–15). Protein–DNA complexes were analysed directly by polyacrylamide gel electrophoresis. **b**, Analysis of hRad52–ssDNA complexes by agarose gel electrophoresis. Reactions used 100 nM ssDNA and the indicated concentrations of hRad52. **c**, Effect of Mg^{2+} on the ssDNA-binding activities of hRad52. Reactions (20 μl) used 100 nM ssDNA and 48 nM hRad52, as described in Methods except that the binding buffer was adjusted to the indicated concentrations of Mg^{2+} . In **b** and **c**, complexes were analysed by agarose gel electrophoresis after protein crosslinking with glutaraldehyde. ^{32}P -labelled DNA was visualized by autoradiography.

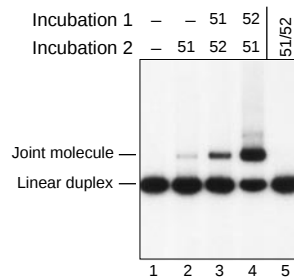


Figure 4 hRad52 is involved in the early stages of hRad51-mediated pairing reactions. Reactions between single-stranded circular and homologous linear duplex DNA were carried out with hRad51 and hRad52 as described in the Fig. 2 legend, except that the proteins were added in a defined order. Lanes 1–4, single-stranded DNA was pre-warmed to 37°C and pre-warmed hRad51 (51) or hRad52 (52) was added as indicated (incubation 1). After 5 min at 37°C, reactions were supplemented with the second pre-warmed protein as indicated (incubation 2), and after a further 5 min at 37°C, the ³²P-labelled linear duplex DNA was added. Incubation was then continued for 2 h. Lane 5, hRad51 and hRad52 were mixed and incubated together for 5 min at 37°C, and then added to the ssDNA. After 5 min incubation at 37°C, ³²P-labelled linear duplex DNA was added and reactions carried out as above. Joint molecules were stabilized and deproteinized and ³²P-labelled DNA was analysed by agarose gel electrophoresis.

hRad51, insufficient to promote joint molecule formation (Fig. 4, lane 2). When 5 μM hRad51 was preincubated with the ssDNA, and the reaction was then supplemented with 0.8 μM hRad52 followed by ³²P-labelled linear duplex DNA, an increase in the efficiency of joint molecule formation was observed (Fig. 4, compare lanes 2 and 3). Under these conditions 15% of the labelled DNA was incorporated into joint molecules. However, 51% of the ³²P-labelled DNA formed joint molecules when hRad52 was incubated first with the ssDNA, followed by addition of hRad51 and the linear duplex (Fig. 4, lane 4). In contrast, preincubation of hRad51 with hRad52, before the addition of the DNA substrates, did not lead to joint molecule formation (Fig. 4, lane 5). Co-incubation of the two proteins under these conditions probably leads to their interaction and inactivation in the absence of a DNA substrate, possibly by the stoichiometric sequestration of hRad52 by hRad51.

Our data show that hRad52 stimulates the formation of joint molecules between homologous single- and double-stranded DNA molecules. These results help us to understand the function of Rad52 in genetic recombination and double-strand break repair. The greatest level of stimulation was observed when hRad51 nucleoprotein filaments were assembled on ssDNA that was first bound by hRad52. The DNA-binding properties of hRad52, and the requirement for hRad52 at an early stage of hRad51-mediated pairing reactions, lead us to suggest that Rad52 facilitates homologous pairing by either: helping to recruit hRad51 to the ssDNA; promoting the formation of extended hRad51 nucleoprotein filaments; or improving the cooperativity of hRad51 such that contiguous filaments cover the entire initiating ssDNA substrate.

It has previously been shown that hRad51 differs from *E. coli* RecA in that it binds both single- and double-stranded DNA *in vitro*^{10,12}, leading to the suggestion that other proteins may be required to help mediate the specific binding of hRad51 to the ssDNA. Our data indicate that hRad51 and hRad52 act together at the ssDNA-binding step that precedes homologous pairing. Analyses of the distribution of Rad51 in yeast, and in mouse and human spermatocytes at meiosis, indicate that it is a component of early recombination nodules, the proposed sites for initiation of strand invasion and chromosome synapsis^{19–22}. Moreover, recent results indicate that Rad51 and Rad52 are found together in *S. cerevisiae*, and that Rad51 focus formation is blocked by *rad52* mutations (S. Gasior and D. Bishop, personal communication), supporting the

idea that Rad52 recruits Rad51 to sites of recombination.

Our results using human Rad52 are in accord with recent observations that *S. cerevisiae* Rad52 protein stimulates homologous pairing and strand exchange reactions catalysed by Rad51 (A. Shinohara and T. Ogawa, personal communication; P. Sung, personal communication; S. Kowalczykowski, personal communication). The way that Rad52 facilitates Rad51-mediated homologous pairing reactions is reminiscent of the way in which the bacteriophage T4 UvsY protein facilitates presynaptic filament formation by UvsX^{23–25}, which is the bacteriophage T4 homologue of RecA/Rad51. This functional similarity between Rad51–Rad52 and UvsX–UvsY indicates a remarkable conservation of recombination functions from prokaryotes to eukaryotic organisms. □

Methods

Purification of recombinant hRad52. Human Rad52 was purified by two methods which yield qualitatively similar proteins. First, hRad52 was purified from 4 litres of *E. coli* FB810 pLysS carrying pFB581 following overexpression in the presence of IPTG (1 mM). Cells were collected, resuspended in 80 ml of T buffer (0.02 M Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 0.02% Triton X-100) containing 5 mM imidazole and lysed by sonication. A high-speed supernatant was loaded onto a 50-ml Talon (Clontech) column equilibrated in T buffer containing 5 mM imidazole. The column was washed sequentially with 25 mM imidazole and 50 mM imidazole in T buffer, and His–Rad52 was eluted with a 50–500 mM imidazole gradient in T buffer. hRad52 was identified by SDS–PAGE, dialysed against R buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 10% glycerol) containing 0.1 M KCl and applied to a 40-ml Affigel–heparin column, which was washed with R buffer containing 0.1 M KCl. hRad52 was eluted with a 0.1–1.0 M KCl gradient in R buffer, dialysed against R containing 0.1 M KCl, and loaded onto a 2-ml ssDNA cellulose column. The column was washed with R buffer containing 0.1 M KCl and eluted with a 0.1–1.0 M KCl gradient in R buffer. hRad52 was stored at –70°C (final yield 3.2 mg). Some hRad52 was lost through precipitation during each dialysis step. In the second method, hRad52 was overexpressed in *E. coli* BL21(DE3) pLysS, and purified as above except that the Affigel–Heparin column was replaced by a Q-sepharose column. When the Q-sepharose was eluted with a 50–500 mM KCl gradient in R, two peaks of hRad52 (~120 and 250 mM KCl) were observed. The first peak, which contained highly purified hRad52, was taken to ssDNA–cellulose as above. The second fraction was impure and was not used further. hRad52 was dialysed and stored in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 50% glycerol and stored at –70°C. hRad52 was essentially free of endo- and exonuclease activities.

DNA-binding assays. The oligonucleotide used in all DNA-binding assays was 5'-TGGGTGAACCTGCAGGTGGGCAAGATGCTCTAGCAATGTAATCGTCAAGCTTATGCCGTT-3'. It was 5' ³²P-labelled using T4 polynucleotide kinase and γ-³²P-ATP. To form duplex DNA it was annealed with its complement: 5'-AACGGCATAAAGCTTGACGATTACATTGCTAGGACATCTTTGCCACCTGCAGGTTACACCA-3'. DNA concentrations are defined in moles of nucleotides. Binding reactions (30 μl) contained ssDNA or dsDNA and hRad52 in 20 mM Triethanolamine-HCl, pH 7.5, 1 mM DTT, 1 mM MgCl₂ and 100 μg ml⁻¹ bovine serum albumin (BSA). Incubation was for 20 min at 37°C. Protein–DNA complexes were analysed by electrophoresis through a 10% PAGE gel using TBE buffer (160 V, 2 h). For analysis by 0.8% agarose gel electrophoresis (80 min at 5.3 V cm⁻¹), complexes were fixed by addition of one-tenth volume of 2% glutaraldehyde followed by 20 min incubation.

Assay for joint molecule formation. hRad51 protein was purified as described²⁶. Single-stranded circular and form I duplex DNA were prepared from phagemid pPB4.3 DNA¹⁰. Duplex DNA was linearized with *Nco*I and 5' ³²P-labelled with polynucleotide kinase and γ-³²P-ATP. Reaction mixtures (10 μl) contained ssDNA (30 μM) and 5' ³²P-labelled linear duplex DNA (10 μM) in 50 mM triethanolamine, pH 7.5, 0.5 mM Mg(OAc)₂, 1 mM dithiothreitol, 2 mM ATP and 100 μg ml⁻¹ BSA. hRad51 and hRad52 proteins were added in 2-μl portions as indicated. For control reactions, 2 μl of an equivalent buffer without protein was added. Reactions were stopped and joint molecules were stabilized and deproteinized by addition of one-fifth volume of 100 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 3% SDS, 5 μg ml⁻¹ ethidium

bromide and 10 mg ml⁻¹ proteinase K, followed by incubation for 20 min at 37 °C. ³²P-labelled DNA products were analysed by 0.8% agarose gel electrophoresis in TAE buffer for 3.5 h at 5.3 V cm⁻¹.

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Stimulation by Rad52 of yeast Rad51-mediated recombination

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In *Saccharomyces cerevisiae*, the *RAD51* and *RAD52* genes are involved in recombination and in repair of damaged DNA^{1–3}. The *RAD51* gene is a structural and functional homologue of the *recA* gene^{4,5} and the gene product participates in strand exchange and single-stranded-DNA-dependent ATP hydrolysis by means of

nucleoprotein filament formation^{6–11}. The *RAD52* gene¹² is important in *RAD51*-mediated recombination^{1–3}. Binding of this protein to Rad51 (refs 4, 13) suggests that they cooperate in recombination. Homologues of both Rad51 and Rad52 are conserved from yeast to humans^{14–16}, suggesting that the mechanisms used for pairing homologous DNA molecules during recombination may be universal in eukaryotes. Here we show that Rad52 protein stimulates Rad51 reactions and that binding to Rad51 is necessary for this stimulatory effect. We conclude that this binding is crucial in recombination and that it facilitates the formation of Rad51 nucleoprotein filaments.

Rad51 catalyses strand exchange between circular single-stranded DNA (ssDNA) and homologous linear double-stranded DNA (dsDNA) to form joint molecules (JM), and open circular duplex DNA molecules (OC)⁸ (Fig. 1a). However, Rad51 is much less active than RecA protein in this reaction, even in the presence of replication protein A (RPA)^{8–11}, indicating that Rad51 may either be inherently inefficient or weakened by a missing cofactor(s).

The effects of Rad52, which binds to DNA (ref. 17, and our unpublished results), and RPA on Rad51-mediated strand exchange were examined using purified proteins in various combinations (Fig. 1b, c). The protein(s) and ssDNA were incubated for 15 min and the strand exchange reactions were started by addition of dsDNA. The ratio of Rad51 to ssDNA used was 3 nucleotides per Rad51 protein and 20 nucleotides per RPA. These ratios were used for all reactions unless otherwise stated and are optimal for formation of nucleoprotein filaments, strand exchange and ssDNA-dependent ATP hydrolysis^{6,8–11}. The Rad51 protein alone showed very weak strand exchange activity as measured by JM formation (Fig. 1b, c, lanes 2). No strand exchange activity was detected for RPA or Rad52 alone, or for both together (Fig. 1b, lanes 3, 4 and 7). RPA or Rad52 stimulated Rad51-dependent JM formation, but not OC (Fig. 1b, lanes 5 and 6). When both Rad52 and RPA were added, OC as well as JM was formed (Fig. 1b, lane 8), indicating that the stimulation of heteroduplex formation requires the presence of both proteins.

When various concentrations of Rad52 were used, a significant enhancement of Rad51-mediated strand exchange was observed only when the Rad52 concentration was over 1 μM (Fig. 1d, e), which corresponds to the ratio of one molecule of Rad52 per 20 nucleotides.

The formation of the product, JM and/or OC, depends on the order of addition of RPA and Rad51 (ref. 18). When RPA was added after preincubation of Rad51 with ssDNA for 5 min, formation of JM was detected at 15 min incubation and OC at 30 min, and the amount of both products increased during a 2-h incubation (Fig. 2a). On the other hand, when RPA was incubated with ssDNA for 5 min before addition of Rad51, formation of JM was enhanced ~3-fold, but few OC were detected (Fig. 2b). The amount of JM was about half that formed when RPA was added before rather than after Rad51. These results indicate that RPA enhances Rad51-mediated strand exchange, but extensive heteroduplex formation, resulting in formation of OC, does not occur when RPA is allowed access to DNA before Rad51. RPA seems to compete with Rad51 for binding sites on DNA, as in the competition between RecA and SSB^{10,19,20}.

We next examined the effect of preincubation of Rad52 with ssDNA on Rad51-mediated strand exchange. When Rad52 was preincubated with ssDNA before adding Rad51, the formation of JM in a 2-h incubation was stimulated to a similar extent as for preincubation with RPA (Fig. 2c), but formation of OC was not significant even after incubation for 6 h (data not shown). Addition of Rad52 after addition of Rad51 stimulated JM formation to a level 1.5-fold higher than that of addition before Rad51, when again no OC was formed (data not shown). On the other hand, when ssDNA was incubated successively for 5 min each with RPA, Rad52 and Rad51 before starting the reaction by adding dsDNA, OC was detected after 60 min incubation and the amount of both JM and

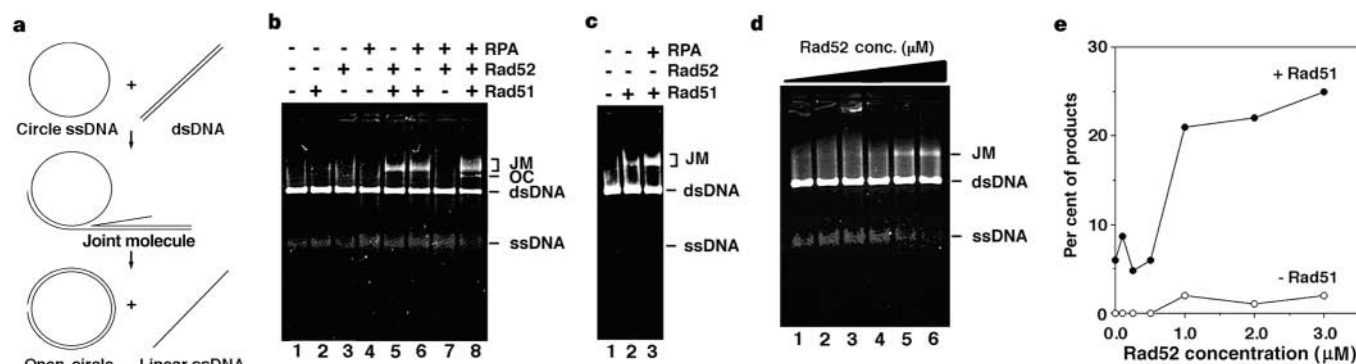


Figure 1 Rad51-mediated strand exchange in the presence of Rad52. **a**, The strand-exchange assay. **b, c**, The strand-exchange reaction was carried out with various combinations of protein(s). Reactions contained ϕ X174 ssDNA (20 μ M), linearized dsDNA (10 μ M), Rad51 (6 μ M), Rad52 (2 μ M), and RPA (1 μ M) proteins, as indicated. Proteins were mixed with ssDNA, incubated for 15 min, and the reactions were started by adding dsDNA and $MgCl_2$. After 2 h (**b**) or 3 h (**c**) incubation, the DNA was deproteinized and analysed by agarose gel electro-

phoresis followed by staining with Syber green. **d**, The strand-exchange reaction was carried out in the presence of Rad51 protein (6 μ M) and various concentrations of Rad52 protein; 0 μ M (lane 1), 0.1 μ M (lane 2), 0.25 μ M (lane 3), 0.5 μ M (lane 4), 1 μ M (lane 5), 2 μ M (lane 6). After 2 h incubation, the DNA was analysed. **e**, Quantification of the result of Rad51-mediated exchange in the presence of various concentrations of Rad52 was done as described in Table 1 legend. An average of three independent experiments is presented; error, ± 2.0 –4.0%.

OC continued to increase as incubation continued (Fig. 2d). About 60% of dsDNA was converted into JM and OC in the presence of both Rad52 and RPA in 2 h, whereas $\sim 30\%$ was converted into JM in the presence of either Rad52 or RPA alone (Table 1). Thus, Rad52 and RPA had additive effects on enhancing the formation of JM and OC. This suggests that Rad52 aids the binding of Rad51 to DNA in the presence of RPA and enhances complete strand exchange.

To test whether Rad52 also stimulates RecA-mediated strand exchange, reactions were carried out in which Rad51 was replaced by RecA. It was found that RPA or SSB from *Escherichia coli* stimulated the reaction (Fig. 2e, lanes 3 and 4), whereas Rad52 did not stimulate the reaction (Fig. 2e, lane 5). The stimulative effects of RPA or SSB were also inhibited by Rad52 (Fig. 2e, lanes 6 and 7). Similarly, a T4 UvsX-mediated strand exchange was stimulated by RPA or T4 gp32, but not by Rad52 (data not shown). These results indicate that stimulation by Rad52 is specific for the Rad51 reaction.

The rate of ATP hydrolysis of Rad51 depends on the length of ssDNA, suggesting that the rate correlates with the formation of nucleoprotein filaments (our unpublished results). By measuring ATPase activity in the presence of Rad52 or RPA or both, the effect of Rad52 on the formation of the Rad51 filament was examined. RPA and Rad52 proteins failed to hydrolyse ATP, regardless of the presence or absence of ssDNA (data not shown). Under optimal conditions (three nucleotides per Rad51), addition of Rad52 slightly enhanced ssDNA-dependent Rad51 ATPase activity (1.2-fold) in the presence of ϕ X174 ssDNA. The enhancement by RPA or SSB was 1.4-fold (Fig. 3a, and results not shown for SSB). On the other hand, at a ratio of 20 nucleotides per Rad51, the ATPase activity of Rad51 was slightly inhibited (0.8-fold) by addition of Rad52 (Fig. 3b), whereas either RPA or SSB inhibited it several fold (Fig. 3c; for SSB,

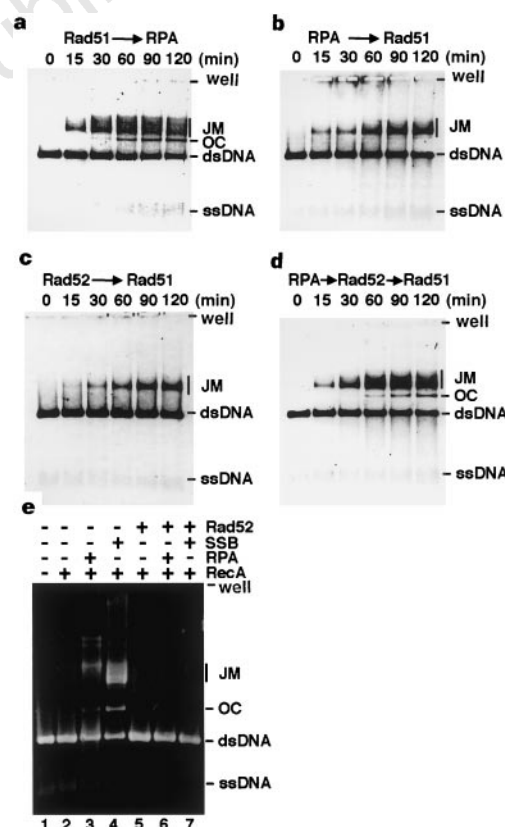


Figure 2 Effect of the order of RPA and/or Rad52 addition on Rad51-mediated strand exchange. Each protein was incubated with ssDNA for 5 min as follows. **a**, RPA was added after Rad51; **b**, RPA was added before Rad51; **c**, Rad52 added before Rad51; **d**, successive incubation of RPA, Rad52 and Rad51 for 5 min each before starting the reaction by addition of dsDNA/ $MgCl_2$. At the indicated times, aliquots were removed, deproteinized and the products analysed in a 0.9% agarose gel. Negative images of each gel are shown. **e**, Effect of Rad52 on RecA-mediated strand exchange. Rad52 (2 μ M), RecA (6 μ M) and RPA (1 μ M) or SSB (0.5 μ M), in the indicated combinations, were successively incubated with ϕ X174 ssDNA (20 μ M) for 5 min each before starting the reaction by addition of dsDNA and $MgCl_2$ and incubated for 30 min. We used a buffer at pH 6.5 for the RecA reaction (the buffer pH is 7.5 in a standard RecA reaction). Under these conditions, the formation of products in the RecA reaction is limited.

Table 1 Effect of Rad52 and RPA on Rad51-mediated strand exchange

Reaction (order)	JM (%)	OC (%)
51	6 $\pm$ 4	ND
52-51	31 $\pm$ 4	ND
RPA-51	32 $\pm$ 4	ND
RPA-52-51	52 $\pm$ 4	6.0 $\pm$ 2

The amount of product formed during a 2-h incubation was determined and the percentage conversion of dsDNA to each product calculated. Gels were analysed by Quantity One software. Percentages are calculated as the intensity of JM or OC product/intensity of (dsDNA + OC + JM products) $\times$ 100. In the case of JM, which is a hybrid molecule of ssDNA and dsDNA, contribution of one ssDNA to the intensity of the band was normalized. An average of three independent experiments is presented. Standard deviation of each analysis was $\pm 4.0\%$ for JM and $\pm 2.0\%$ for OC. ND, not detectable.

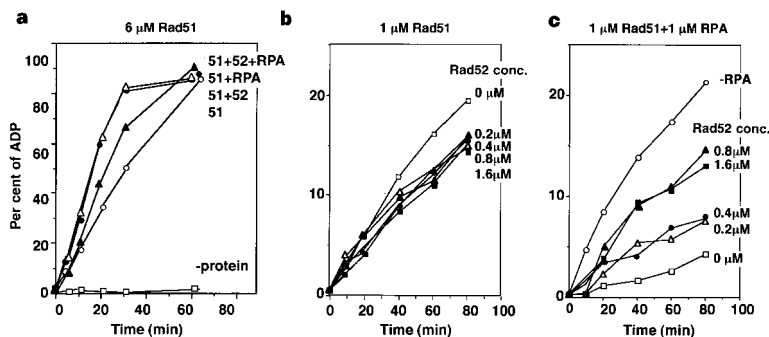


Figure 3 Effects of Rad52 and RPA proteins on Rad51 ATPase activity. **a**, Rad51 ATPase was measured at an optimal ratio of DNA to Rad51 of 3 nucleotides. The reaction contained various combinations of Rad51 (6.6 μM), Rad52 (2 μM), RPA (1 μM), ssDNA (20 μM) and 0.2 mM 32 P- α -ATP, as indicated. ϕ X174 ssDNA (20 μM) was used as a cofactor. Without protein (squares); Rad51 (open circles); Rad51 and RPA (filled circles); Rad51 and Rad52 (filled triangles); Rad51, Rad52 and RPA (open triangles). **b**, **c**, Rad51 ATPase was examined at a ratio of Rad51 to poly(dT) DNA (average length, 200 nucleotides) of 20 nucleotides. Rad51 (1 μM) alone (**b**),

and Rad51 (1 μM) and RPA (1 μM) (**c**, except for open circles) were mixed with various concentrations of Rad52 protein: 0 μM (open squares), 0.2 μM (open triangles), 0.4 μM (filled circles), 0.8 μM (filled triangles), 1.6 μM (filled squares) of Rad52 protein. The reaction was initiated by addition of ATP. Open circles in **c** indicate ATP hydrolysis by Rad51 in the absence of Rad52 and RPA. The average of three independent experiments is presented; error was ± 2.0 –3.0% for each point. Similar results were obtained when poly(dT) was replaced by ϕ X174 ssDNA (data not shown).

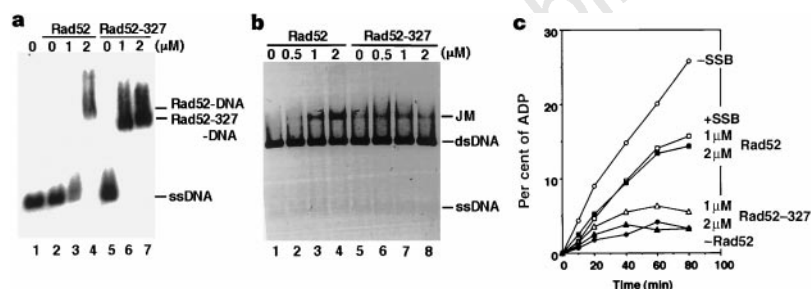


Figure 4 Effect of Rad52-327 protein on Rad51 activity. **a**, Gel shift analysis of the Rad52-ssDNA complex. 20 μM of 32 P-labelled (dT)₆₀ oligonucleotide was incubated with various concentrations of Rad52 and Rad52-327 proteins. The products of the reactions were applied to a 1.0% agarose gel. **b**, Strand-exchange reactions were performed in the presence of Rad51 and various concentrations of Rad52 or Rad52-327 proteins. **c**, The Rad51 ATPase at a ratio of nucleotides to

Rad51 of 20 was measured with various combinations of Rad51, Rad52 or Rad52-327, SSB and poly(dT) (20 μM) as for Fig. 3c. Rad51 alone (open circles); Rad51 and SSB (filled circles); Rad51, SSB and 1 μM Rad52 (open squares), 2 μM (filled rectangles); Rad51, SSB and 1 μM Rad52-327 (open triangles), 2 μM Rad52-327 (filled triangles). The concentrations of Rad51 and SSB were 1 μM and 0.5 μM, respectively.

see Fig. 4c). The inhibition of Rad51's ATPase by SSB or RPA has been reported¹⁰. When Rad52 is included in the reaction, the inhibitory effect of RPA is alleviated (Fig. 3c). This relief of inhibition is maximal at a ratio of Rad52 to Rad51 of ~ 1 (Fig. 3c). The rate of ATP hydrolysis by Rad51 in the presence of Rad52 was not affected by the presence of RPA (Fig. 3b, c). These results indicate that Rad52 helps the binding of Rad51 to ssDNA precoated with RPA.

The carboxy-terminal region of Rad52 is required for the interaction with Rad51 (ref. 13). The mutant carrying a C-terminal-truncated protein, *rad52-327* which has 327 amino acids starting from the N terminus and is lacking 177 C-terminal residues, is defective in both DNA repair and recombination, although the strain retains residual activity for repair of damaged DNA^{13,21}. The Rad52-327 protein bound to ssDNA as well as the wild-type protein¹⁷ in a gel-shift assay using a 32 P-labelled p(dT)₆₀ oligonucleotide (Fig. 4a). However, the mutant protein was several-fold less active in stimulating the Rad51-mediated strand exchange reaction (Fig. 4b). Furthermore, the Rad52-327 protein enhanced Rad51's ATPase 1.5-fold in the presence of SSB (Fig. 4c) or RPA (data not shown), whereas the wild-type Rad52 enhanced it ~ 8 -fold. These results show that the C-terminal region of Rad52, which interacts with Rad51, is required for efficient stimulation of Rad51 activity.

We have described the roles of Rad52 and RPA in the *in vitro*

Rad51 reaction, particularly the cooperation of these proteins in the pairing of homologous DNAs. It has been proposed that the stimulation by RPA after formation of nucleoprotein filaments is due to its ability to remove the secondary structures of ssDNA^{9,10}. However, prior access of RPA to ssDNA inhibits the Rad51 reactions and this inhibition is alleviated by Rad52 (Figs 2b, 3c). Both stimulation of the Rad51 reaction and alleviation of the inhibition by Rad52 are specific to the Rad51 reaction, and this specificity resides in the specificity of its interaction with Rad51. Our results suggest that Rad52 facilitates formation of Rad51 filaments in the presynaptic phase of recombination. Stimulation of JM formation in a human Rad51 strand-exchange reaction^{22,23} by human Rad52, which binds to human Rad51 (refs 24, 27), and the presence of Rad51 foci on yeast meiotic subnuclear sites of wild type, but not of the *rad52* deletion mutant (D. Bishop, personal communication), are in agreement with our results. For other reports of Rad51 stimulation by Rad52 see refs 28, 29. Although recombination is a complex process involving other gene products¹⁻³, the Rad51 reactions assisted by Rad52 are likely to be central to the molecular process of homologous recombination in eukaryotes. □

Methods

Proteins. Rad51 protein was purified from *E. coli* as described⁶. Rad52 protein was purified from *E. coli* 594 strain as described elsewhere²⁵. Yeast RPA protein was purified from RDY2275, a yeast overproducer of yeast RPA (ref. 26; provided by R. Kolodner). *E. coli* SSB protein was purchased from Pharmacia.

Conditions for strand-exchange reaction. We first incubated Rad52 or RPA protein with ϕ X174 viral DNA (final concentration, 20 μ M in nucleotide) for 5 min at 37 °C in 9 μ l buffer (20 mM HEPES, pH 6.5, 1 mM DTT, 6.6 mM $MgCl_2$, 3 mM ATP, 20 mM creatine phosphate, 0.1 mg ml⁻¹ creatine kinase, 50 μ g ml⁻¹ BSA), then Rad51 protein (0.5 μ l of 123 μ M) was added. After 5 min of further incubation, 0.1 μ g of *Pst*I-linearized ϕ X174 dsDNA in 1 μ l and 100 mM $MgCl_2$ in 1 μ l were added to start the reaction. After incubation at 37 °C for the indicated times, SDS and proteinase K were added to final concentrations of 0.5% and 0.5 mg ml⁻¹, respectively, followed by a 20-min incubation. Samples were mixed with 1 μ l dye solution (0.01% of bromophenol blue, 20% glycerol) and analysed on a 0.9% agarose gel prepared in 1 $\times$ TAE buffer (40 mM Tris-acetate, pH 7.5, 0.5 mM EDTA). Electrophoresis was for 15 h at 1.2 V cm⁻¹ at 4 °C. The gel was stained with 0.1 μ g ml⁻¹ of Syber green (Molecular Probe). The image of the gel was recorded with a CCD camera (Epi-UV FA1100, Aisin Cosmos); densitometric analysis was carried out using Quantity One software (PDI). Images were processed using Photoshop (Adobe). In the case of the RecA reaction, Rad51 was simply substituted by RecA (6 μ M).

Assay for ssDNA-dependent ATP hydrolysis. ATP hydrolysis was analysed in 10 μ l of buffer containing 20 mM HEPES, pH 6.5, 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM ³²P- α -ATP, 50 μ g ml⁻¹ BSA, 20 μ M ssDNA at 37 °C. The reaction was started by addition of the indicated amounts of Rad51 protein. At the indicated time, 1- μ l aliquots were removed and directly spotted on a PEI paper. The PEI papers were developed in 0.85 M potassium phosphate (pH 2.8). Radioactive ATP and ADP spots were analysed by using the phosphorimager BAS2,000 (Fuji Film).

Gel shift assay of Rad52-p(dT)₆₀ complexes. 20 μ M (in nucleotide) of p(dT)₆₀ oligonucleotide whose 5' end was labelled with ³²P was incubated with Rad52 or Rad52-327 proteins in 10 μ l buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 50 μ g ml⁻¹ BSA) at 37 °C for 10 min. Samples were analysed on a 1.0% of agarose gel in 0.1 $\times$ TAE. Electrophoresis was done at 10 V cm⁻¹ for 90 min.

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Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A

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The generation of a double-strand break in the *Saccharomyces cerevisiae* genome is a potentially catastrophic event that can induce cell-cycle arrest or ultimately result in loss of cell viability. The repair of such lesions is strongly dependent on proteins encoded by the *RAD52* epistasis group of genes (*RAD50-55*, *RAD57*, *MRE11*, *XRS2*)^{1,2}, as well as the *RFA1*^{3,4} and *RAD59* genes⁵. *rad52* mutants exhibit the most severe phenotypic defects in double-strand break repair², but almost nothing is known about the biochemical role of Rad52 protein. Rad51 protein promotes DNA strand exchange^{6–8} and acts similarly to RecA protein⁹. Yeast Rad52 protein interacts with Rad51 protein^{10,11}, binds single-stranded DNA and stimulates annealing of complementary single-stranded DNA¹². We find that Rad52 protein stimulates DNA strand exchange by targeting Rad51 protein to a complex of replication protein A (RPA) with single-stranded DNA. Rad52 protein affects an early step in the reaction, pre-synaptic filament formation, by overcoming the inhibitory effects of the competitor, RPA. Furthermore, stimulation is dependent on the concerted action of both Rad51 protein and RPA, implying that specific protein–protein interactions between Rad52 protein, Rad51 protein and RPA are required.

As with the RecA protein¹³, assembly of a presynaptic filament composed of single-stranded (ss)DNA coated with Rad51 protein is a first step in DNA strand exchange. Secondary structure in ssDNA imposes a barrier to nucleoprotein filament continuity which RPA can overcome through its ability to destabilize duplex DNA^{14,15}. Although RPA is required for optimal DNA strand exchange and ATP hydrolysis by Rad51 protein, because of their shared ability to bind ssDNA they are potential competitors: when RPA binds ssDNA first, a long delay in ATP hydrolysis (T.S., unpublished observation) and a substantial decrease in the amount of DNA strand exchange results¹⁶. Thus, displacement of RPA is necessary to form an active presynaptic complex¹⁵. Double-stranded DNA (dsDNA) is a substrate in DNA strand exchange but, as it is readily bound by Rad51 protein, it can also be a competitor of presynaptic filament formation⁷. Because both RPA and dsDNA are present *in vivo*

during Rad51 protein–ssDNA assembly, some mechanism presumably exists to allow filament formation in the presence of these competitors.

To examine the impact of Rad52 protein on Rad51 protein-promoted DNA strand exchange, Rad52 protein was purified from *Escherichia coli*. Figure 1 shows DNA strand exchange reactions in which ssDNA was incubated with RPA for one minute before addition of Rad51 and Rad52 proteins. DNA strand exchange was initiated by addition of dsDNA at 1, 5 or 15 min after the addition of these proteins. Product formation is stimulated significantly by Rad52 protein (Fig. 1a, lanes 3, 5 and 7); at longer times (30 min), stimulation occurs, but the relative magnitude decreases, because Rad51 protein can slowly displace RPA (data not shown). Stimulation is not due to Rad52 protein-mediated DNA strand exchange because the reaction is completely dependent on Rad51 protein (Fig. 1a, lane 1). The Rad52 protein concentration is optimal for these reactions: halving its concentration reduces the yield by approximately half, whereas increasing it by 50% increases it negligibly (data not shown). These results parallel other recent

results¹⁷. Figure 2 shows the reaction time course. Rad52 protein increases the yield of paired products, but it has little impact on the progress of the reaction. These results indicate that Rad52 protein acts by increasing the amount of an essential DNA strand exchange participant, and that it does so by antagonizing the inhibitory effect of prebound RPA on the presynaptic phase of DNA strand exchange: we presume that Rad52 protein either increases dissociation of RPA and/or assembly of Rad51 protein.

To determine whether Rad52 protein moderates sequestration of Rad51 protein by dsDNA, Rad51 protein was incubated with dsDNA either in the simultaneous or subsequent presence of Rad52 protein; these DNA pairing reactions were completely blocked (data not shown), demonstrating that Rad52 protein does not reverse the inhibitory effect of Rad51 protein binding to dsDNA. Taken together, these results suggest that Rad52 protein facilitates the loading of Rad51 protein onto RPA–ssDNA.

The need for interaction between Rad52 protein and RPA, as previously suggested^{4,18}, was evaluated by substituting a ssDNA-binding protein for RPA. *E. coli* SSB protein, when added after Rad51 protein, stimulates its activity¹⁵; but when SSB protein is allowed to bind ssDNA before Rad51 protein is added, no product forms (Fig. 3). However, Rad52 protein cannot overcome this inhibition, even after 15 min of co-incubation, (Fig. 3, lanes 2 and 3) suggesting that a specific interaction between Rad52 protein and RPA is necessary for stimulation.

If Rad51 protein simply occupies a ssDNA site that is vacated by RPA, then RecA protein might act similarly; in contrast, if a Rad52–Rad51 protein interaction were essential, then RecA protein should not be stimulated by Rad52 protein. When RecA protein is substituted for Rad51 protein (Fig. 4), DNA strand exchange is efficient, even when RPA is allowed to prebind the ssDNA (Fig. 4, lane 2). However, when Rad52 protein is added (Fig. 4, lane 3), product formation is inhibited. Additionally, Rad52 protein completely blocks DNA strand exchange in an otherwise identical RecA protein reaction that used SSB protein instead of RPA (data not shown). From these experiments we conclude that the Rad52 protein–RPA interaction is not sufficient to stimulate RecA protein, and that the Rad51–Rad52 protein interaction is necessary for stimulation by Rad52 protein.

The potential of ssDNA-binding proteins to inhibit as well as to stimulate the activity of DNA strand exchange proteins underscores the need for proper coordination of the recombination machinery for efficient function. In this regard, the Rad51–Rad52–RPA system is like that of the bacteriophage T4 UvsX–UvsY–gene 32 protein recombination proteins, in which UvsY will stimulate DNA strand

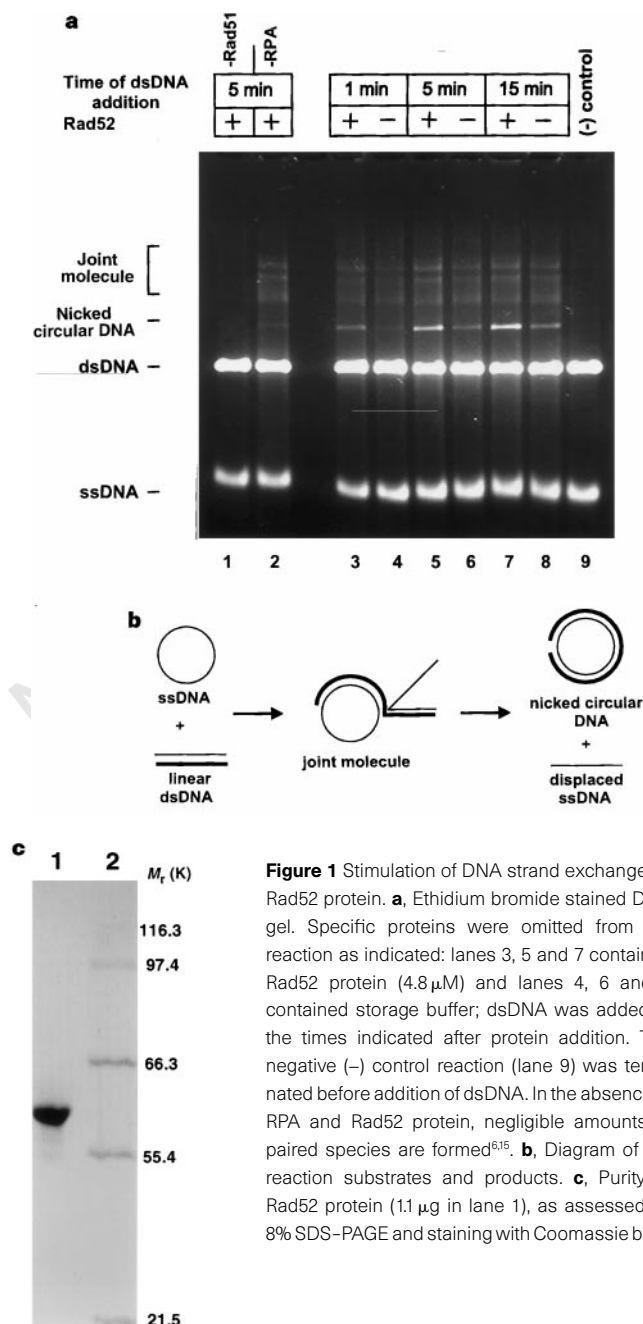


Figure 1 Stimulation of DNA strand exchange by Rad52 protein. **a**, Ethidium bromide stained DNA gel. Specific proteins were omitted from the reaction as indicated: lanes 3, 5 and 7 contained Rad52 protein (4.8 μ M) and lanes 4, 6 and 8 contained storage buffer; dsDNA was added at the times indicated after protein addition. The negative (–) control reaction (lane 9) was terminated before addition of dsDNA. In the absence of RPA and Rad52 protein, negligible amounts of paired species are formed^{6,15}. **b**, Diagram of the reaction substrates and products. **c**, Purity of Rad52 protein (1.1 μ g in lane 1), as assessed by 8% SDS–PAGE and staining with Coomassie blue.

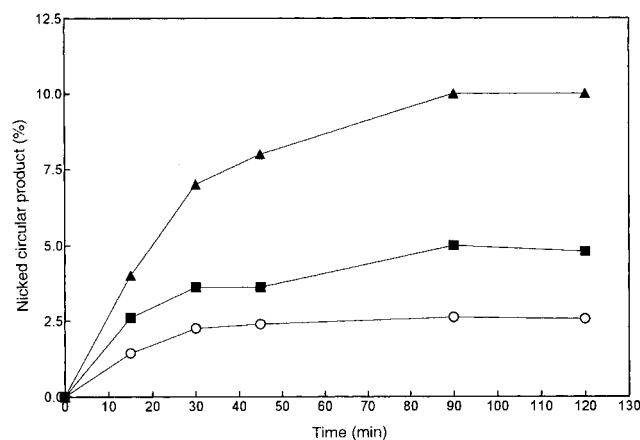


Figure 2 Time course for DNA strand exchange initiated at various times after addition of Rad52 protein. RPA was allowed to pre-bind ssDNA for 5 min before addition of Rad51 and Rad52 proteins, and DNA strand exchange was initiated by addition of dsDNA 1, 5 or 15 min after protein addition: Circles, 1 min; squares, 5 min; triangles, 15 min.

exchange by UvsX using a preformed gene 32 protein–ssDNA complex¹⁹. Like Rad51 protein, UvsX binds both dsDNA and ssDNA⁹, and, like Rad52 protein, UvsY interacts with the strand exchange protein UvsX and the ssDNA-binding protein gene 32 protein²⁰. Although the activities of Rad52 protein described here are important in double-strand DNA break repair and homologous recombination, they cannot completely account for all of the roles of *RAD52* in these processes. Other studies have suggested additional *RAD51*-independent functions for *RAD52* (refs 21, 22): whether or not these are dependent on the intrinsic ability of Rad52 protein to renature complementary ssDNA, on other as-yet

undefined biochemical activities, or on additional protein factors remains to be investigated. □

Methods

Strains and plasmids. BL21(DE3) Δ *recA* was constructed by E. Zaitsev of this laboratory, and BLR containing pLysS was from Novagen. PEZRad51 (Rad51 protein overexpression) and pEZRad52/3 (Rad52 protein overexpression) were constructed by cloning of the *RAD51* and *RAD52* (from the third ATG codon) ORFs into pET3a and pET21a (Novagen), respectively. Constructs were confirmed by sequence analysis.

Rad52 protein purification. A 12-litre culture of BL21(DE3) Δ *recA* containing pEZRad52/3 was grown at 30 °C in LB broth (Ap; 100 g ml⁻¹) and induced at $A_{600} = 1$ (IPTG, 0.1 mM final concentration) for 1 h. Cells were resuspended in 5 volumes of lysis buffer (450 mM NaCl in M₂₀DEG buffer (50 mM MES (pH 6.5), 1 mM DTT, 1 mM EDTA, 10% glycerol)); lysed by passage through a French press at 4 °C; and PMSF (1 mM), benzamide (10 mM), and pepstatin A (1 μ g ml⁻¹) added. After a low-speed spin (6,000 r.p.m., 10 min), the supernatant was loaded onto Q-Sepharose (35 ml). Ammonium sulphate (0.29 g ml⁻¹) was added to the flow-through fraction and, following dialysis of the pellet against lysis buffer, the solution was diluted 3-fold with M₂₀DEG buffer and applied to a P-11 phosphocellulose column (35 ml). Peak fractions (eluting at 400 mM NaCl in M₂₀DEG buffer) were diluted 2-fold with M₂₀DEG buffer and loaded onto ssDNA–cellulose (50 ml) which was washed with steps of 0.3 M and 0.6 M NaCl in M₂₀DEG buffer. The last wash was applied to a hydroxyapatite column (4 ml) and fractionated with a 10–200 mM potassium phosphate gradient in 0.2 M NaCl in M₂₀DEG buffer. Rad52 protein eluted at 90 mM potassium phosphate, was made 1 M in NaCl, dialysed against 1 M NaCl in T₂₀DEG buffer (20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 10% glycerol) and loaded onto Superose-12 (25 ml). Rad52 protein, which eluted in the void volume, was dialysed against storage buffer (175 mM NaCl in T₂₀DEG buffer) and stored at –80 °C. Protein concentration was determined using an extinction coefficient of 2.43×10^4 at 280 nm. Approximately 1–2 mg purified protein was recovered from 10 g of cells. The identity of the protein was confirmed by immunoblotting.

Rad51 protein and RPA. Rad51 protein was purified as described⁶, but with addition of a Cibacron-blue chromatography step (Rad51 protein eluted at 1 M KCl) and omission of gel filtration. RPA was purified as described¹⁵.

DNA strand exchange. Reactions (12.5 μ l) contained 42 mM MOPS (pH 7.4), 3 mM magnesium acetate, 1 mM DTT, 25 μ g ml⁻¹ BSA, 2.5 mM ATP, and 33 μ M (nucleotides) Φ X174 ssDNA (New England Biolabs). RPA (1 μ M) or SSB protein (18 μ M) was added and incubated at 37 °C for 1 min, at which time Rad51 (13 μ M) and Rad52 (3.2 μ M) proteins were added. After further incubation for the indicated times, *Pst*I-linearized Φ X174 dsDNA (33 μ M nucleotides) and spermidine acetate (4 mM) were added. Reactions were terminated after 90 min, or as indicated otherwise, and analysed¹⁵.

RecA protein reactions were similar, except that the ATP concentration was 1 mM, an ATP regeneration system was included²³, dsDNA was added 1 min after addition of the last protein, and spermidine acetate was omitted.

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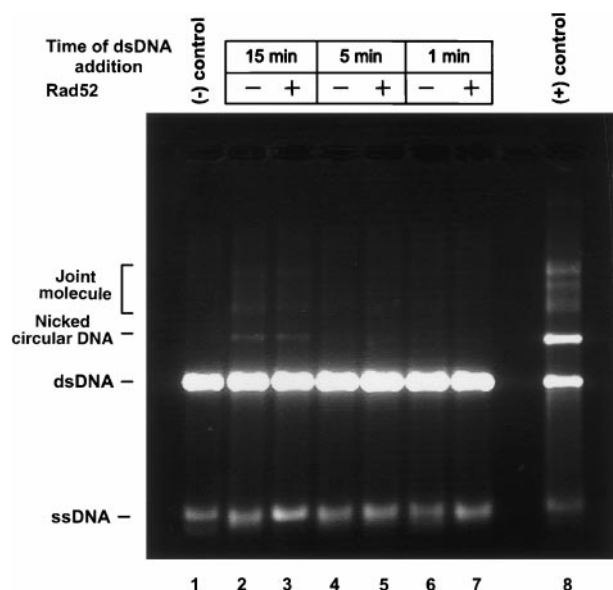


Figure 3 Inhibition of DNA strand exchange by SSB protein is not overcome by Rad52 protein. Standard reactions were performed, except that RPA was replaced by SSB protein (18 μ M). Rad52 protein was added to reactions as indicated (lanes 3, 5 and 7), and dsDNA was added after Rad52 protein or buffer at indicated times. The negative (–) control reaction (lane 1) was the same as for Fig. 1. In the positive (+) control reaction (lane 8), the reaction was staged so that Rad51 protein was assembled on ssDNA first and, after a 15-min incubation at 37 °C, SSB protein was added; 15 min later, dsDNA was added to initiate the reaction.

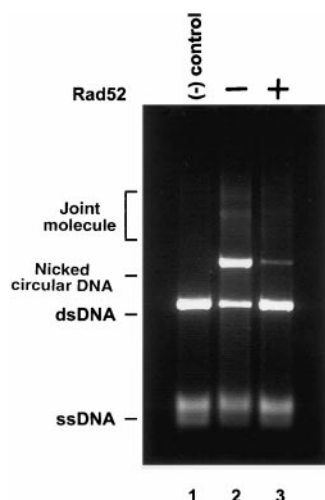


Figure 4 Rad52 protein does not stimulate *RecA* protein-promoted DNA strand exchange. Standard reactions containing RPA were supplemented with an ATP regeneration system, and Rad51 protein was replaced by *RecA* protein (10 μ M). Rad52 protein or buffer was added (lanes 3 and 2, respectively) in the standard manner.

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Crystal structure of p50/p65 heterodimer of transcription factor NF- κ B bound to DNA

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The NF- κ B p50/p65 heterodimer is the classical member of the Rel family of transcription factors which regulate diverse cellular functions such as immune response, cell growth, and development^{1–3}. Other mammalian Rel family members, including the proteins p52, proto-oncoprotein c-Rel, and RelB, all have amino-terminal Rel-homology regions (RHRs)^{4–7}. The RHR is responsible for the dimerization, DNA binding and cytosolic localization of these proteins by virtue of complex formation with inhibitor κ B proteins⁸. Signal-induced removal of κ B inhibitors allows translocation of dimers to the cell nucleus and transcriptional regulation of κ B DNA-containing genes⁹. NF- κ B specifically recognizes κ B DNA elements^{1,10,11} with a consensus sequence of 5'-GGGRNYYCC-3' (R is an unspecified purine; Y is an unspecified pyrimidine; and N is any nucleotide). Here we report the crystal structure at 2.9 Å resolution of the p50/p65 heterodimer bound to the κ B DNA of the intronic enhancer of the immunoglobulin light-chain gene. Our structure reveals a 5-base-pair 5' subsite for p50, and a 4-base-pair 3' subsite for p65. This structure indicates why the p50/p65 heterodimer interface is stronger than that of either homodimer. A comparison of this structure with those of other Rel dimers reveals that both subunits adopt variable conformations in a DNA-sequence-dependent manner. Our results explain the different behaviour of the p50/p65 heterodimer with heterologous promoters.

The overall structure of the p50/p65 heterodimer is consistent with that of other Rel family proteins (Fig. 1a)^{12,13,31}. Each subunit

consists of two immunoglobulin-like domains connected by a 10-amino-acid flexible linker. Dimers form through a β -sheet sandwich of the carboxy-terminal dimerization domains. Unlike most DNA-binding proteins, which use α -helices for base-pair recognition, Rel family dimers use loops from the edges of the N- and C-terminal domains to mediate DNA contacts (Fig. 2a). Secondary structures of the subunits are equivalent, apart from a 32-amino-acid insert in the N-terminal domain of p50 that adds a second α -helix (Fig. 1b) (to simplify description, p50 residues will be written in normal type and p65 residues will be written in italics).

Dimerization in the heterodimer is localized to the C-terminal domains and consists of a hydrophobic core stapled by various polar interactions (Fig. 2b). The backbones of the dimerization domains are highly similar and superimpose with a root-mean-square deviation (r.m.s.d.) of 1.10 Å. The buried surface area upon dimerization is 1,442 Å². Of particular interest is the hydrogen bond between homologous residues Asp 254 of p50 and Asn 200 of p65, which is unique in the heterodimer. In the homodimers, an interaction between homologous residues is energetically unfavourable because this juxtaposes two like charges. Also of interest are nine polar contacts, most of which occur between the backbones of hydrophobic core residues and the side chains of hydrogen-bonding residues. These results are consistent with the observation that the affinity between homodimers of p50 and p65 is weaker than for the heterodimers¹⁴.

The co-crystallized DNA consists of 11 base pairs with a complementary overhanging base on each end (Fig. 1c). The 3' ten base pairs of the target DNA define the immunoglobulin(Ig) κ B element. Analysis of the central 11 base pairs with the program CURVES¹⁵ revealed a 14-degree bend. The bases T₀ and G₋₁ are also twisted out of plane owing to this DNA bending. This DNA bending agrees with biochemical experiments indicating that the p50/p65 heterodimer induces a 17-degree bend in H2- κ B DNA¹⁶.

Previous experiments have indicated that the p50 subunit preferentially occupies the 5' end of an Ig- κ B target DNA^{2,10,14}. The heterodimer crystal structure not only confirms this orientation, but also demonstrates the exact positioning (Fig. 3a). The observed base-specific contacts support the idea that the Ig- κ B site consists of a 5-base-pair 5'-GGGAC-3' subsite contacted by p50, and a 4-base-pair 5'-TTCC-3' subsite contacted by p65. Upon binding to DNA, the heterodimer buries 3,754 Å² of the total solvent-accessible surface area, of which 58% is derived from interaction with p50. Overall, the DNA contacts mediated by p50 and p65 in the heterodimer structure are similar to those in the homodimer structures^{12,13}.

Base-specific binding by p50 to the subsite occurs through the residues Arg 54, Arg 56, Tyr 57, Glu 60, His 64 and Lys 241 (Fig. 3b, top). The strict conservation of the first three guanines in the p50 subsite is determined by the hydrogen bonds made by His 64 with the N7 group of G₋₅, and by Arg 56 and Arg 54 with both the N7 and O6 groups of G₋₄ and G₋₃. This interaction is strengthened by Glu 60, which hydrogen-bonds with these arginines and makes base-specific contacts to the N4 groups of C₋₅ and C₋₄. Tyr 57 makes van der Waals contacts with C₋₃ and T₋₂ and is itself strongly

Table 1 Data collection and structure refinement statistics

Data collection						
Resolution	Total reflections	Unique data	R_{merge}	I/σ (last shell)	Completeness (last shell)	
2.9 Å	88,449	26,430	0.082	15.8 (3.1)	97.2 (88.6)	
Refinement						
Resolution (Å)	Reflections ($F_o > 2\sigma$)	Atoms protein/DNA	R factor (%) [*]		$R_{\text{m.s.d.}}$	
			R_{work}	R_{free}	Bonds (Å)	Angles (°)
8.0–2.9	19562	4629/486	21.0	32.1	0.014	1.97

^{*}*R*_{work} = $\sum ||F_o| - |F_c|| / \sum |F_o|$, *R*_{free} = $\sum ||F_o| - |F_c|| / \sum |F_o|$, where *T* is a test set containing a randomly selected 5% of the observations omitted from the refinement.

restrained by many van der Waals interactions. The bulk of the Tyr 57 side chain results in an absence of purines in the -2, top-strand position in the observed κ B sites. The requirement for the nucleotide at position -1 is more relaxed because this base hydrogen-bonds with the flexible Lys 241.

Binding to the 3' subsite by the p65 subunit occurs through Arg 33, Arg 35, Tyr 36, Glu 39 and Arg 187 (Fig. 3b, bottom). The base conservation observed in κ B DNA sequences at positions +3 and +4 is a result of the hydrogen bonds formed with Arg 33 and Arg 35. Glu 39 strengthens this conservation by hydrogen-bonding to the N4 groups of C₊₃ and C₊₄. Unlike its p50 counterpart Lys 241, the

geometry of Arg 187 is limited by interactions with Glu 39, and thus contacts the O4 groups of T₊₂. Therefore, Glu 39 defines the base conservation by buttressing these three arginines and forming a highly ordered tetrad. Tyr 36, which is constrained by protein contacts in a manner similar to Tyr 57, makes van der Waals contacts to the thymines at positions +1 and +2. Again, a potential steric hindrance with guanines at these sites explains the presence of thymines in most of the known κ B sites.

Both p50 and p65 make extensive contacts with the ribose phosphate backbone of DNA: p50 makes 16 and p65 makes 12. Notably, the backbone nitrogens of Gly 65 and Gly 66 make phosphate

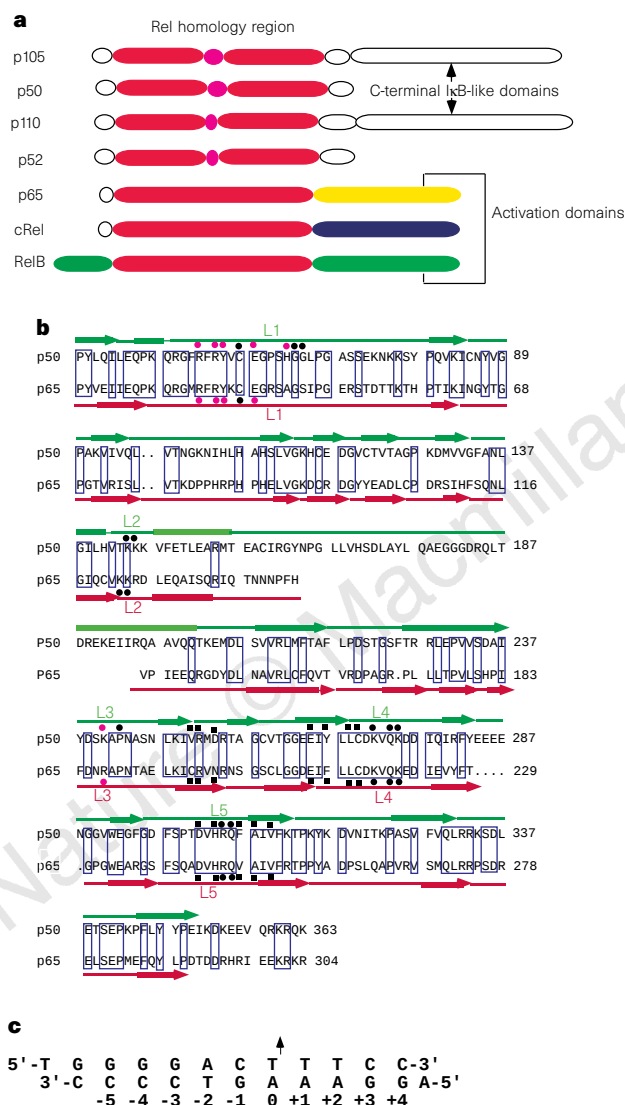


Figure 1 The Rel/NF- κ B family of proteins. **a**, Sequence motifs. p50 and p52 are proteolysis products of p105 and p110, respectively, in which the inhibitor (I) κ B-like domains are removed. Regions of high homology exist among the family members (RHR) (red ovals). p105(p50) and p110(p52) also contain 32- and 18-amino-acid inserts, respectively, in the RHR (purple ovals). Non-homologous transactivation domains exist in p65, c-Rel, and RelB (different coloured ovals). **b**, Primary sequences and secondary structures of the RHR in mouse p50 (green) and p65 (red) including β -strands (arrow), α -helices (bars), and identical residues (blue boxes). A 32-amino-acid insert exists in p50 after residue 137 in p65. L1-L5 are DNA-contacting loops. Residues that contact DNA (pink circles), contact the DNA backbone (black circles) and contribute to the dimer interface (black squares) are indicated. **c**, The co-crystallized DNA containing the 10-base-pair κ B element from the intronic enhancer of the immunoglobulin κ light-chain gene. The two subsites are separated by a pseudodyad T:A base pair (arrow).

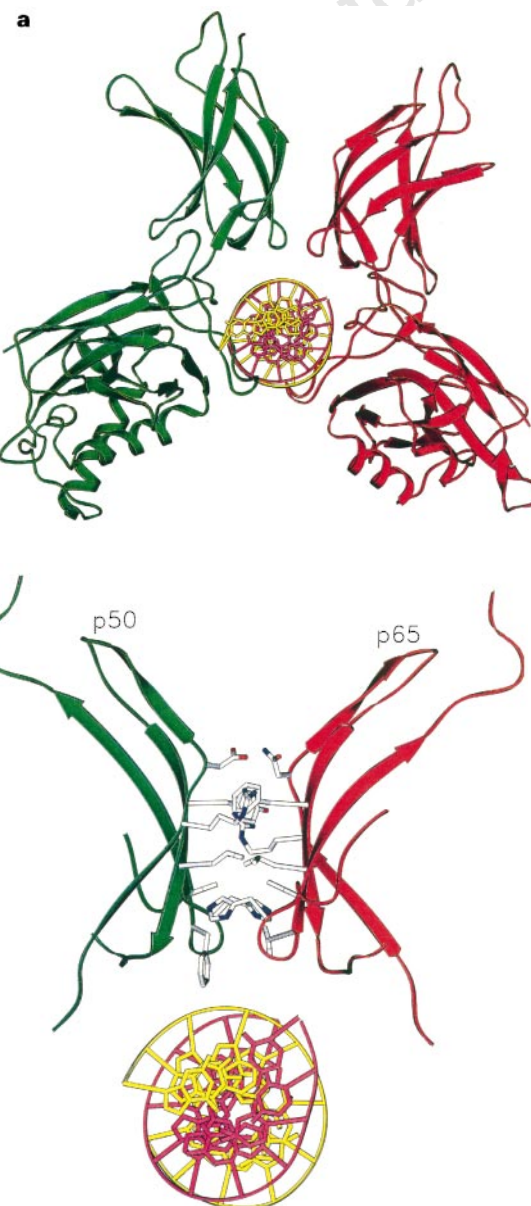


Figure 2 The structure of the heterodimer bound on the I κ B DNA. **a**, Ribbon drawing of the entire complex viewed down the DNA helical axis. The p50 subunit is in green and the p65 subunit is in red. The top strand of DNA is in pink, and the bottom strand is in yellow. Drawing produced with SETOR³⁰. **b**, The hydrophobic core of the dimer interface between p50 (green) and p65 (red) consists of an array of nonpolar hydrocarbons, aromatic rings and uncharged polar residues pointing from the β -sheets in towards the interface. Only the β -strands and seven residues contributing to the interface are displayed. Oxygens (red) and nitrogens (dark blue) are included.

contacts to G₋₆ and G₋₅ and allow His 64 to make proper base-specific contacts. The presence of a side chain at Gly 65 has been found to disrupt DNA binding¹⁷. Lys 144 and Lys 145 in p50 and the homologous residues in p65, Lys 122 and Lys 123, are the only residues that contact DNA in the minor groove. Pro 189 also makes van der Waals contacts to the DNA between the deoxyribose moieties of C₋₁ and A₋₂. Unlike most contacts made by p65, the position of this contact does not exactly mirror the one made in p50 by Pro 243, but is shifted (Fig. 3a). This deviation probably reflects global movements that p65 undergoes to bind this DNA.

The p50/p65 heterodimer has a particularly high DNA affinity compared to most eukaryotic transcription factors: between 10⁻¹³ to 10⁻¹⁰ M (refs 18, 19). This affinity cannot be accounted for by direct DNA readout alone as other transcription factors make similar numbers of contacts. Three other sources might contribute to this high binding affinity. First, interdomain interactions in both subunits might provide a high degree of cooperativity between the two DNA-contacting domains. These interactions result in an inter-domain solvent-excluded area of 757 Å² for the p65 subunit and 612 Å² for the p50 subunit. Second, although the target DNA consists of two subsites, phosphate contacts beyond subsite limits could provide coupling energy in the protein-DNA binding pathway²⁰. Finally, the extensive dimer interface could also generate large cooperativity in DNA binding.

Dimerization domain superposition of the p50/DNA half-complex from the heterodimer onto that from the human p50 homodimer reveals that the N-terminal domains require 9 degrees

rotation and 2.3 Å translation for superposition¹³. A similar rotation and 1.4 Å translation of the N-terminal domain of p65 is needed when compared to subunit A of the p65/DNA half-complex from the homodimer³¹. The largest component of this rotation occurs around an axis almost parallel to the DNA long axis. Consequently, the base-contacting residues of both subunits move slightly from their optimum positions on DNA.

The p50/p65 heterodimer is the most abundant of the Rel/NF-κB dimers and plays a more elaborate role than other factors in regulating gene expression^{2,3}. The p50/p65/Ig-κB DNA complex has served as an archetype for Rel family homo- and heterodimer interaction with DNA targets. Our crystal structure now provides structural information regarding the binding modes of biologically relevant κB DNA targets that do not reflect the observed consensus^{10,21,22}. Whereas the 5' p50 subsite is strictly conserved as 5'-GGGRN-3', the 3' p65 subsite is not as well conserved. Conformational adjustments in both subunits are probably necessary for base-specific binding to these κB targets.

Despite architectural differences in the DNAs used for co-crystallization with Rel dimers^{12,13}, each sequence is bound in a way that preserves the protein-DNA interface chemistry. The subunits' linker regions, in response to the DNA sequence, can adjust the N-terminal domain so that the DNA-contacting residues are aligned properly. Consistent with our observations, the p50/p65 heterodimer has been shown to bend the κB targets H2-κB and IFN-κB, which differ at only three nonconserved bases, by 7 and 17 degrees, respectively¹⁶. Furthermore, these κB targets do not function in the

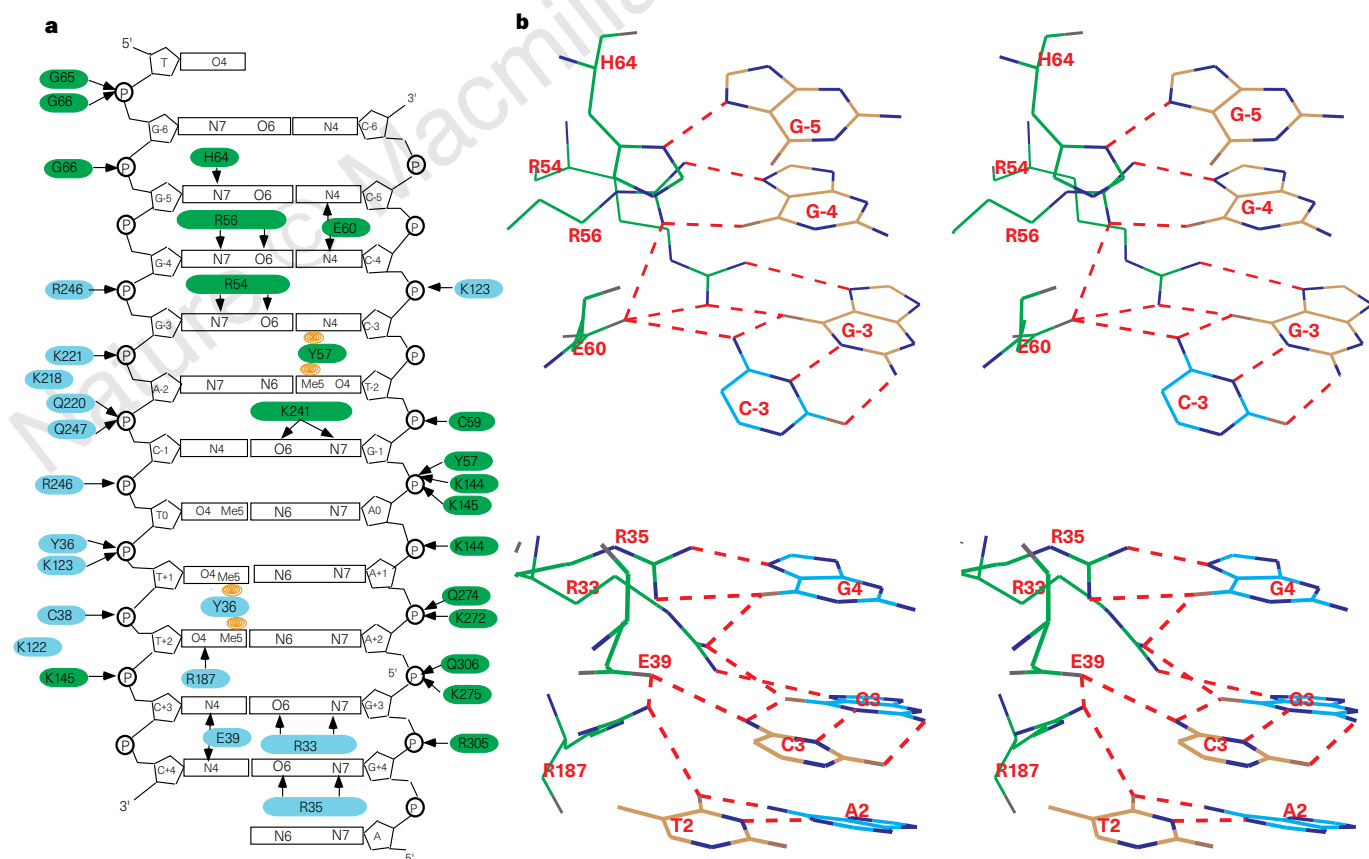


Figure 3 DNA contacts made by the heterodimer. **a**, The DNA contacts made by the p50/p65 NF-κB heterodimer. Blue and green distinguish the p65 and the p50 subunits, respectively. Arrows denote hydrogen bonds; brown ovals indicate van der Waals contacts. The p50 subunit binds to the 5' five-base-pair subsite; the p65 subunit binds to the 3' four-base-pair subsite. **b** (top), Stereo pair of the base-specific interactions mediated by Arg 54, Arg 56, Glu 60 and His 64 of the p50 subunit. Hydrogen bonds are drawn as dashed lines between grey oxygens and dark blue nitrogens. p50 residues are green, top strand bases are yellow, while the bottom strand base is in blue. Bottom, Stereo pair of the base-specific interactions mediated by Arg 34, Arg 35, Glu 39 and Arg 187 of the p65 subunit. p65 residues are shown in green.

same manner *in vivo* when placed in heterologous promoters as in their cognate promoters²³. The combined results of structural, *in vitro* bending, and *in vivo* transcription experiments therefore suggest that the sequence and flexibilities of different DNA targets determine the final conformation of the complexes in a manner sensitive to small differences¹⁸. □

Methods

Protein purification. Truncated murine p50 (residues 39–364) and p65 (residues 19–291) were overexpressed separately in *E. coli* cells with a T7 promoter driven expression plasmid (Novagen) and purified independently over S-Sepharose (Pharmacia) and Sephadex-75 columns (Pharmacia). The two proteins were combined with a slight molar excess of p65 and unfolded in denaturing buffer (0.5 M NaCl, 7 M urea, 0.5 mM EDTA, 0.1 mM PMSF, 10 mM β -mercaptoethanol, 25 mM Tris-HCl, pH 7.5). The protein was then refolded by dialysis into 20 mM NaCl and 25 mM Tris-HCl, pH 7.5. The renatured heterodimer was purified once again over an S-Sepharose column, concentrated, aliquoted, sorted at -80°C until needed, and used within 5 h of thawing.

DNA purification. Two 12-base-pair oligonucleotides were synthesized, 5'-TGGGGACTTCC-3' and 5'-AGGAAAGTCCCC-3', by phosphoramidite synthesis. After deblocking, the oligonucleotides were purified over a Q-Sepharose column. Peak fractions were pooled, buffered to 50 mM MES, pH 6.0, desalted, and concentrated to 2 mM in a final buffer of 10 mM Tris-HCl, pH 7.5. Equimolar amounts of both strands were mixed and annealed. The double-stranded oligonucleotide was mixed in 10% molar excess of the p50/p65 heterodimer.

Crystallization and data collection. Crystals grew in nine days at 18°C ($0.2 \times 0.2 \times 0.4$ mm) from 6- μl hanging drops in a final concentration of 6 mg ml⁻¹ complex, 50 mM sodium acetate, pH 5.5, 100 mM CaCl₂, 0.125% β -octyl-glucoside, 1 mM spermine, 10 mM dithiothreitol, and 8% polyethylene glycol 3350. Before data collection, crystals were dialysed for two days at 18°C in 25 μl of 50 mM sodium acetate, pH 5.6, 100 mM CaCl₂, 0.125% β -octyl-glucoside, 10 mM dithiothreitol, 1 mM sodium spermine and 15% polyethylene glycol 3350, against 5 ml of a cryosolvent consisting of the same components plus 30% glycerol. Crystals were then mounted in nylon loops and flash-frozen in a liquid nitrogen stream. X-ray diffraction data were collected at 105 K using a Marresearch imaging plate system and X-rays from a large Rigaku rotation anode RU operated at 50 kV and 100 mA. Unit cell dimensions are: $a = b = 106.61$, $c = 206.56$ Å, $\alpha = \beta = \gamma = 90.0^{\circ}$. The space group is $P4_32_12$. There is one dimer in the asymmetric unit, with a solvent volume fraction of 0.59. Data were indexed and integrated using DENZO and scaled by SCALEPACK²⁴. Data collection and reduction statistics are summarized in Table 1.

Structure solution and refinement. The structure of the heterodimer was solved by molecular replacement using X-PLOR²⁵. The search model consisted of subunit A from the homodimeric p65/DNA complex and the monomer of human p50/DNA complex. Molecular replacement results showed that $P4_32_12$ is the correct space group. Patterson correlation refinement revealed two solutions: a translation search yielded an *R* factor 0.46 for solution (1) and 0.48 for solution (2).

The orientation and position of the two solutions were subsequently refined by rigid-body refinement using 10.0 to 3.5 Å data. Refinement was performed first on the whole molecule, then the two subunits, and finally by allowing each domain to move independently. This resulted in an *R* factor of 0.39 for solution (1) and 0.47 for solution (2). Hence, solution (1) was chosen as a starting model. Successive cycles of positional refinement and simulated annealing in X-PLOR combined with model rebuilding progressively improved the structure. Manual adjustments were made based on $|2F_o - F_c|$ difference maps on SGI graphics workstations using programs TOM²⁶ and O (ref. 27). The parameter set of Engh and Huber²⁸ was applied for the structure refine-

ments. Inclusion of individual isotropic temperature factors in the refinement resulted in a final *R* factor of 0.21 and an *R*_{free} of 0.32. Ramachandran plots generated with PROCHECK²⁹ showed that 96% non-glycine residues were in the most favoured regions (70%) and additional allowed regions (26%) in both subunits. Of the 1% residues with ϕ and ψ angles in disallowed regions, all are located in the loop areas. The final refinement statistics and stereochemical parameters are presented in Table 1.

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Correspondence and requests for materials should be addressed to G.G. (e-mail: gghosh@chem.ucsd.edu). Coordinates have been deposited in Brookhaven Protein Data Bank under accession code 1VKX.

Products of science

Kicking off this eclectic collection is a new higher capacity 'smart' card — other items of interest include a microscope for birefringence imaging, an assortment of astronomy tools and a real-time LC/FTIR instrument.

SmartMedia

From Toshiba

Technology that doubles the capacity of the company's removable storage cards while retaining their small size and simplicity

These are the first commercial cards from Toshiba to use technology that results in a capacity of 16 megabytes, twice that of the previous generation of cards. The new cards are scheduled to debut in the first quarter of 1998. They represent a highly compact, light and cost-effective data storage for personal digital media. They are just over the size of a postage stamp, and use a NAND flash memory chip to support high-speed rewriting of individual memory sectors. A control circuit within the NAND chip treats two separate chips as a single chip, while the company's mounting technology allows the two chips to be assembled on a single printed circuit board and pasted into the base card. The result is doubled capacity in a base card with exactly the same dimensions and contact area as the original card. There are 2-, 4- and 8-MB SmartMedia cards available, each incorporating a single NAND chip. The higher capacity will also support such applications as taking higher resolution images in future generations of digital camera.

Reader Enquiry No. 100

Biacore probe

From Biacore

An analysis system designed for biomolecular interaction analysis and improved isolation and purification of biomolecules

The mobile probe is contained within a modified pipette, allowing easy aspiration of sample solution. It is connected by a fibre-optic cable to the base unit, which contains the power supply, detection system and LCD panel. The biological activity of samples can therefore be monitored directly at each stage of isolation or purification. The immediate evaluation of results enables the user to identify quickly the relevant fractions for further processing. Removable heads may be



Biacore's mobile biomolecular probe.

stored, making the instrument ideal for a multi-user environment where a variety of samples are being handled. The probe is said to save significant time over such applications as PAGE, HPLC or ELISA. The system uses touch-screen software control, which guides the user through the analytical steps.

Reader Enquiry No. 101

Microscopic perspective

MetaPolScope

From Universal Imaging

A birefringence imaging microscope with the hardware and software required for control and analysis

Birefringence imaging is a new contrast enhancement technique that can be used to visualize microscopic structures with improved resolution, sensitivity and speed. It converts polarized light microscopes into quantitative retardance imaging systems. Using MetaPolScope to control acquisition and analysis, a user can quantify magnitudes and angles of birefringence for biological samples, optical components, plastics, metals, fibres, as well as petrography and chemical crystallography samples. Unlike polarized light microscopy or differential interference contrast (DIC) microscopy, birefringence imaging is performed irrespective of specimen orientation. This product is based upon the PolScope developed by Rudolf Oldenbourg at the Marine Biological Laboratory (*Nature* 381, 811–812; 1996), Woods Hole, Massachusetts, and uses precision liquid crystal variable retarders from Cambridge Research and Instrumentation.

Reader Enquiry No. 102

BioProbe

From Park Scientific Instruments

A powerful scanning probe microscope (SPM) for the life sciences

BioProbe is a complete SPM system that mounts easily onto the stage of all popular inverted and upright microscopes. It is said to be the only SPM/optical microscope that allows researchers to utilize all optical and scanning probe techniques simultaneously. Users can view their samples with optical contrast methods, such as phase-contrast, Nomarski/DIC, and polarization while simultaneously acquiring high-resolution SPM images. The microscope features a full complement of SPM modes for operation in air or liquid, including contact, intermittent-contact, and non-contact atomic force microscopy, lateral force microscopy, force modulation microscopy, phase detection



BioProbe from Park Scientific.

microscopy and magnetic force microscopy. Additionally, operation of BioProbe will not inhibit the ability of the optical microscopes to perform such techniques as fluorescence, scanning-confocal, bright-field, and dark-field microscopy. The system allows samples to be easily mounted using standard microscope slides, cover slips or Petri dishes, and can be imaged in air or a variety of liquids, including buffers, physiological fluids and organic solvents.

Reader Enquiry No. 103

Microbiology

Virusolve II

From Anachem Bioscience

This is a new disinfectant cleaner to help maintain pipettes and combat contamination

Virusolve II can be used as a general multi-purpose disinfectant, but is formulated to be effective against viruses, bacteria and fungi, and to reduce risk of cross-infection. It is said to be completely safe, non-corrosive, non-hazardous and environmentally friendly. This disinfectant is available in three packaging options: as concentrate, as a ready-to-use spray or as disposable wipes.

Reader Enquiry No. 104

RABIT

From Don Whitley Scientific

A Windows-based system designed for the rapid automated bacterial impedance technique (RABIT)

The dedicated CPU of earlier versions has been replaced with a high-specification PC fitted with a special integral data logging card. This upgraded hardware is stated to result in significantly improved software performance. Options for data manipulation have been extended to include a facility for exporting data to spreadsheet software and include the ability to edit worksheets. There is 'at-a-glance' status display of all connected modules, and an option to select and view



Run RABIT to detect and enumerate bacteria.

any cell location with a single mouse movement. This instrument is designed to be flexible and modular, and to provide rapid, automatic indication of the presence and numbers of bacteria in a wide range of samples. Up to 32 reusable cells can be incubated in a single module and the system can be expanded to up to 16 modules, giving a capacity of 512 individual tests.

Reader Enquiry No. 105

Scan RDI

From Chemunex

Faster microbial detection and identification with results down to a single cell, within 90 min of sampling, states Chemunex

By utilizing an air-cooled laser, the Scan RDI microbial detector can be used wherever there is access to a standard mains electrical socket; there is no need for water or plumbing. To support the rapid identification of microorganisms, such as *Cryptosporidium*, *Escherichia coli* and coliforms, a wide range of probes and antibodies is under development. The system combines high-speed laser scanning and sophisticated cell labelling. This avoids the need for a time-consuming, cell-multiplication step and provides ultra-sensitive results. According to the manufacturer, the speed of the laser analysis is such that samples can be loaded every three minutes. In addition to fungi, bacteria and even spores, stressed and fastidious cells can all be detected and counted through the use of complementary Fluorassure reagents. Direct cell detection means there is no need for complex statistical interpretation of the data. Data output is in the form of a tabulated display/printout. This includes calibration data, a complete results dossier and statistical data for trend analysis. The laser unit is linked via a flexible fibre optic cable, allowing the laser to be situated under the bench, if required.

Reader Enquiry No. 106

Astronomy

Research telescope range

From Telescope Technologies

A variety of fully automated/robotic telescopes and a full range of associated instrumentation

The range features research-class astronomical telescopes of 1–5-m diameter,

which can be configured for optical or infrared astronomy and have an alt/az or alt/alt design. Special designs are also available for optical interferometry. Robotic control systems allow unattended operation on remote sites or pre-scheduled long-term surveys. Mirror coating plants for 1–8-m diameter mirrors allow for the use of aluminium, silver or composite coatings. Instrumentation available with this range includes CCD cameras, spectrographs and infrared cameras. Telescope specifications can be obtained from the company's website at www.telescopes.demon.uk.

Reader Enquiry No. 107

UV transmission filter

From Pixel Vision

Developed by Ofil, this ultraviolet transmission filter is stated to attenuate solar light efficiently

The solar blind filters are manufactured using a polymer-based process and are stated to achieve greater than ten per cent transmission in the ultraviolet spectral region and greater than ten orders of magnitude rejection in the visible spectral region. The filters are designed for coupling to photomultiplier tubes, image intensifier tubes and ultraviolet-sensitive, solid-state sensors. The efficient rejection of solar light permits sensors to accomplish ultraviolet sensing, which is uncorrupted by solar light. Ultraviolet detection can be used for such applications as flame detection, plume phenomenology, pollution detection, and high-voltage line coronas characterization.

Reader Enquiry No. 108

Scientific Astronomer

From Wolfram Research

The calculating powers of Mathematica turns skyward with software that determines which constellations, planets, galaxies and comets are visible from a given observing location

There are said to be extensive two- and three-dimensional graphic capabilities within this system, enabling detailed charting of anything from regional star charts and planispheres to three-dimensional planetary views. The package determines optimal dates for the best naked-eye view of specific objects, including stars, low-orbit satellites and the asteroid Vesta. It contains searchable information about the properties and movements of over nine thousand stars and other objects. The package is fully programmable and allows users to add further astronomical information. Five types of star charts are defined, including two wide-field charts that allow the user to zoom in to any portion of the sky. All of the charts can show star spectral colours, mesh lines, a skyline, the horizon line and the Milky Way. Scientific

Astronomer provides for labelling constellations, stars, planets, deep-sky objects, and so on. Planet plotting is done in two- and three-dimensional forms with surface features for the Earth, the Moon, Mars and Jupiter shown on the plots. Moons and their shadows are displayed for both the Earth and Jupiter. The satellite tracking features can help to create track plots, make visibility predictions, and project satellite tracks onto star charts — users can even track the space station Mir. The program is Mathematica 3-compatible, has an easy-to-use interface with palettes and buttons, and is fully integrated into Mathematica's comprehensive help browser system. It runs on both Windows and Unix platforms; a Macintosh version will soon follow.

Reader Enquiry No. 109

Lab and field

Nichiryo NSP-7000

From CP Instruments

A multichannel autosampling system that is designed to undertake repetitive tasks in busy laboratories using microtitration plates

The Nichiryo NSP-7000 offers dispensing, serial dilution, plate-to-plate sample transfer, interplate transfer and mixing facilities in a single, easy-to-programme instrument. Stated to be a fraction of the cost of a fully robotic sampling system, the NSP-7000 autosampler will accommodate sample volumes from 5 to 210 µl in 1-µl increments. Protocols are stored in menu-driven files that are easy to programme. Moreover, multistage protocols can be chained together in a single streamlined sequence programme.

Reader Enquiry No. 110

Pour Boats

From Clark Scientific

A new weighing boat that has a bow for easy pouring and dispensing

These disposable weighing boats are moulded polystyrene and incorporate a pouring spout. This means there is no need to flex or bend the boat to ensure quick and complete transfer of liquids or solids. Unlike many standard boats these benefit from a special formulation of antistatic



Pour Boats put the bow on weighing boats.

polystyrene. However, they are also available in a high impact, standard polystyrene material. The pour boats are manufactured with a smooth flat base, so they sit firmly on the balance pan. They are available in capacities from 30 ml to 100 ml with a colour choice of black or white. Packs of 500 for the 30-ml size and 250 for the 100-ml size are available.

Reader Enquiry No. 111

SunWize portable energy system

From SunWize Technologies

A portable solar power system for portable electronic devices, such as a lap-top computer

Featuring a high-efficiency solar panel just larger than a legal pad, the system can also be used to operate or recharge low-power products, such as a cell phone, a two-way radio, lap-top computers, and the like. It is designed for people who need to use computers in places where there is no utility power or where power sources are unreliable. It is designed to be a rugged product, and works anywhere where there is direct or strong sunlight. According to the manufacturer, the portable energy system will accommodate all major brands of PC and Macintosh portable computers. In its basic configuration, the system consists of a solar panel, a voltage controller, interconnection cables and a storage case. When used with one panel, the system delivers enough sustained electrical power to the computer, up to 9 W, to double or triple the run time of a portable computer operating with battery power, or it can recharge the battery when the computer is turned off. By connecting a second panel, this power system generates sufficient sustained power to run the computer and simultaneously charge its battery. Two output ports on the controller send power either to the portable computer or to a second device. Built into the case is a LCD meter that measures sunlight intensity and allows optimum placement of the panel. The system weighs in at just over 900 g and measures about 40 cm × 27 cm × 2 cm.

Reader Enquiry No. 112

Racksys tube storage system

From Racksys

A storage system for 1.5–2-ml reaction tubes

A system that consists of one tray with two drawers, Racksys can be integrated into every refrigerator, including the 4 °C and –20 °C compartments. The installation is extremely simple and fixes the system tightly into the refrigerator. More than 700 tubes can be stored and arranged systematically, saving on a great deal of space. After pulling out the drawer the reaction tubes can be easily identified and handled. The drawers are protected against dropping out



Chilling in the rack with Racksys tube storage.

of the system. There are also racks for work with reaction tubes — 16 of these racks can be used in the system instead of the standard storage racks.

Reader Enquiry No. 113

Tipster

From Bellco Glass

A unit that has been designed to eliminate the need to load plastic pipette tips manually into tip containers

The Tipster automatically loads six templates of tips per container, which are then transferred by the pipette tip picker to tip holders. This device offers a peg and grommet attachment to fit Bellco orbital shakers, and is made with lightweight materials, which are said to assure impact resistance for safe operation. This unit also accommodates most brands of tips, and is easy to maintain. The Tipster includes one main box, a set of six templates, a collection tray, a template holder one picker with a holder, and an anti-skid frame that can be mounted temporarily or permanently.

Reader Enquiry No. 114

J-Lite JLA-8.1000 rotor

From Beckman Instruments

Integrated rotor and centrifuge system for large-scale batch pelleting

Compatible with the Avanti J-20 series centrifuges, the new rotor can process six litres of cell cultures in less than ten minutes. The composite rotor system features removable canisters and is said to be well suited for processing large volumes of cells, subcellular organelles and proteins. The rotor's 17-kg weight is less than that of traditional 6 × 500-ml rotors. The polycarbonate and polypropylene bottle systems hold one litre each and their wide-mouth design makes filling and emptying the bottles quick and easy. Beckman has designed a spatula that is contoured to the bottle dimensions to facilitate pellet removal. The canister design features a bottle/canister closure system that enhances sample containment and eliminates the need for a second closure cap. The bottle cap/closure seals quickly and efficiently and acts as a secondary canister seal.

Reader Enquiry No. 115

Slide Caddy

From ISC

A medical technologist from North Carolina has invented an improved and safer method of organizing slides, according to ISC

Slide Caddy is a durable unit that keeps up to 75 slides in order so that they are easy to access. It minimizes the amount of space needed to store slides while maximizing the user's ability to locate particular samples. Multiple caddies connect securely to create a larger storage unit with a variety of shapes and sizes, allowing a large quantity of slides to be moved quickly, with reduced risk of breakage. The system should facilitate numerical indexing, colour coding, as well as other organizational systems.

Reader Enquiry No. 116

Platelet incubator/shaker

From Lab-Plant

A new platelet incubator/shaker for low-cost handling of blood products

Platelets have traditionally been agitated at room temperature, but due to improved standards it is now generally required that an ambient temperature of 22 °C be maintained. The incubator shaker holds up to 12 platelet packs and the shaking action is asymmetrical with an angle of 10° and a speed range of 2–50 r.p.m. The asymmetrical action is good for platelet packs, ensuring constant slow movement of the contents. The refrigerated incubator maintains set temperatures of 18–22 °C, even in hot ambient conditions.

Reader Enquiry No. 117

Chemistry

MicroBeta Jet

From EG&G Wallac

A liquid scintillation and luminescence counter with reagent injectors for flash luminescence

This counter is suitable for use with all types of luminescence reagents, either flash-type or glowing. The new instrument is available in

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READER ENQUIRY NO. 23

one-, two-, three- or six-detector models. The single-detector model can have up to four injectors per detector; whereas, multi-detector models can have up to two injectors per detector. MicroBeta counters are said to provide a compact, complete solution for laboratories needing quantitative detection of γ - or β -emitting nucleides or luminescence markers. According to the manufacturer, these systems are well suited for samples labelled with ^3H , ^{14}C , ^{32}P , ^{33}P , ^{35}S , ^{51}Cr and ^{125}I , and especially for processing scintillation proximity assays. The MicroBeta design allows simultaneous counting with photomultiplier tubes both above and below the sample to enhance measurement sensitivity. MicroBeta counters accept samples in tubes or on filters, as well as all commercially available plates. The maximum capacity, using standard 96-well plates, is 1,536 or 3,072 samples.

Reader Enquiry No. 118

Low-temperature thermoluminescence

From Louis Rey

A process developed to help assess the structure of liquids at ultra-low doses

In this procedure, a solid substance is submitted to strong irradiation with high-energy photons or particles so as to 'activate' it. In this process, many electrons belonging to its internal structures are elevated from their original sites into excited levels where they may sit in stable traps for long periods. However, when some outside energy — heat or light — is beamed onto this material the traps will empty and the electrons will go back to their ground state, producing a significant glow. This is well known for natural (rocks or sand grains) or man-made materials (ceramics), which have been submitted to natural radiation, including cosmic rays, for centuries or even millennia and which can be dated with accuracy by their light emission when rapidly heated to high temperatures (350 °C). This phenomenon, thermoluminescence, is in current use for archaeological

research and for monitoring personnel working on nuclear sites. Liquids, in their frozen state, behave in the same way and display thermoluminescence when they are progressively heated up after a strong activation in the solid phase. Activation is generally accomplished by using γ rays or electron beams at liquid nitrogen temperature (near 77 K). The resulting thermoluminescence spectra give reliable information on the starting fluids. Most often, the luminescent centres coincide with 'defects' in the crystalline network that, in turn, are dependent upon given structural patterns pre-existing within the original liquid. Recent experiments done on pure water and on deuterium oxide have demonstrated distinctive features which are highly reproducible if all the operating conditions are well controlled. Applied to ultra-low doses, the same technology shows different reproducible spectra in Fig. 1. This might help and discriminate the starting solutions and, maybe, help understand their particular structures (Fig. 2). (For more information fax to Switzerland: 41 21 652 0964.)

Reader Enquiry No. 119

LC/FTIR system

From Bourne Scientific

Described by the manufacturer as what may be the first real-time LC/FTIR system

The system operates in real time by passing the output of the LC through an ultrasonic nebulizer to a desolvation tube, where a vacuum chamber is used to evaporate the solvent. The residue from each peak is deposited onto a moving Zn/Se window. The FTIR, whose sample beam is focused on the window, creates a spectrum for each peak. It can classify or even identify the components of each chromatographic peak and, as such, can serve as a supplement or even an alternative to LC/MS. For example, IR is probably the best method for identifying which of several isomers are contained

in a particular peak. The system includes the vacuum interface with specialized optical components for depositing and scanning the sample, the FTIR spectrometer and the data system for processing the spectra. With an additional module, the operator can modify the LC/FTIR for use as a detector for GC; the changeover takes about an hour. It can also be used with SFC (supercritical fluid chromatograph) instruments.

Reader Enquiry No. 120

AMB 110 and AMB 210 balances

From Adam Equipment

Moisture content analysis for a multitude of research and quality-control applications

AdamLab offers two new moisture balances: the AMB 110 and AMB 210 (100-g and 210-g capacities). Both should enable the user to determine, rapidly and accurately, the moisture content of a sample and to display the result either as a percentage of the original weight or as a percentage of the resultant dry weight (± 0.1 per cent). These latest units incorporate expanded software capabilities giving the user more control of the test parameters and a choice of seven analysis modes. The sample is dried by infrared lamps until the moisture has been driven off, or for a time specified by the user. The digital LED display on the balance shows the per cent moisture content or dry weight, and time elapsed, continuously updating itself as the test progresses. Drying time and temperature are user selectable, with the balance automatically regulating temperature to within 1 °C. Standard equipment includes an RS-232 interface, which enables printer output, as well as allowing a computer record to be kept.

Reader Enquiry No. 121

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

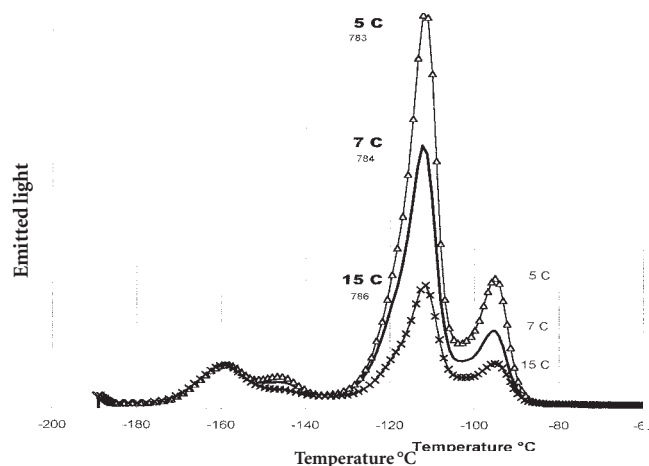


Figure 1 Thermoluminescence of D_2O and H_2O after γ irradiation (30 kGy) at -196°C .

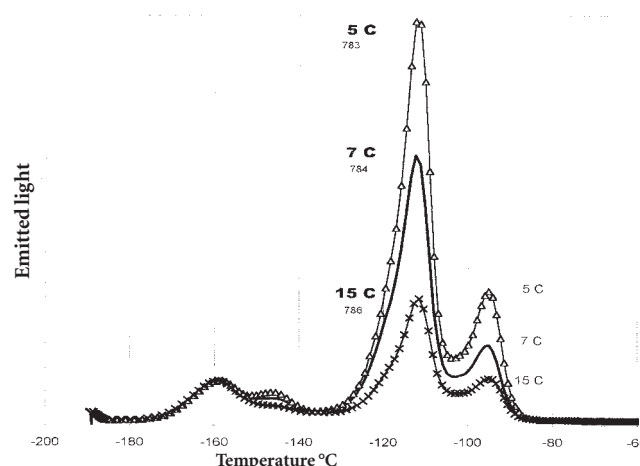


Figure 2 Thermoluminescence of ultra-low doses of NaCl in H_2O after γ irradiation (30 kGy) at -196°C .